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# Selection of glyphosate resistance in *Amaranthus tuberculatus* (Mq ex DC) J.D. Sauer and potential for transfer of glyphosate resistance in *Conyza*

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**Selection of glyphosate resistance in *Amaranthus tuberculatus* (Mq. ex DC)**

**J.D. Sauer and potential for transfer of glyphosate resistance in *Conyza***

by

**Ian A. Zelaya**

A thesis submitted to the graduate faculty  
in partial fulfillment of the requirements for the degree of  
**DOCTOR OF PHILOSOPHY**

**Major: Crop Production and Physiology**

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Iowa State University  
Ames, Iowa  
2004

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**DEDICATION**

Two special persons passed away during my stay at Iowa State University, this dissertation is dedicated to the memory of Evangelina Rubio–Argüeta and Lorna I. Zelaya (QDDG).

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## LIST OF SYMBOLS AND NOMENCLATURE

**ae:** acid equivalent

**ALS:** acetolactate synthase (EC 2.2.1.6)

**AMPA:** aminomethylphosphonic acid

**bp:** base pair(s)

**DAHP:** 2-keto-3-deoxy-D-arabinoheptulosonate-7-phosphate

**DAHPS:** DAHP synthase (EC 4.1.2.15)

**DPM:** disintegrations per minute

**E4P:** erythrose-4-phosphate

**EPSP:** 5-O-(1-carboxyvinyl)-3-phosphoshikimate

**EPSPS:** 3-phosphoshikimate 1-carboxyvinyltransferase (EC 2.5.1.19)

**GA<sub>3</sub>:** 90% (+)-gibberellic acid

**GOX:** glyphosate oxidase reductase

**HPLC:** high pressure liquid chromatography

**IC<sub>50</sub>:** dose inhibiting enzyme activity by 50%

**kb:** kilobase(s)

**kDa:** kilodalton(s)

**K<sub>i</sub>:** dissociation constant

**K<sub>M</sub>:** Michaelis-Menten constant

**LSS:** liquid scintillation spectroscopy

**M<sub>r</sub>:** relative molecular mass

**NMR:** nuclear magnetic resonance spectroscopy

**<sub>N</sub>TPS:** amino-terminal transit peptide sequence

**ORF:** open reading frame

**pEPSPS:** EPSPS preprotein

**PEP:** phosphoenolpyruvate

**P<sub>i</sub>:** inorganic phosphate

**PPO:** protoporphyrinogen oxidase (EC 1.3.3.4)

**S3P:** shikimate-3-phosphate

## ABSTRACT

Iowa farmers often use glyphosate (*N*-(phosphonomethyl) glycine) in glyphosate resistant crops for weed management. Inconsistent glyphosate control has been reported in common waterhemp (*Amaranthus tuberculatus* (Mq. ex DC) and horseweed (*Conyza canadensis* (L.) Cronq.) populations. Therefore, an investigation was undertaken to ascertain the potential for selection of glyphosate resistance in common waterhemp, evaluate the genetics, and assess the potential for resistance transfer in two *Conyza* species.

Rate responses verified that 0.62 kg acid equivalents (ae) of glyphosate ha<sup>-1</sup> were required to reduce biomass by 50% ( $GR_{50}$ ) in common waterhemp from Everly, Iowa, compared to 0.24 kg ae ha<sup>-1</sup> of a pristine population from Paint Creek, Ohio. Recurrent selection was performed to isolate resistant and susceptible biotypes within the Everly population. Rate responses suggested that the frequency of resistant individuals increased in the first ( $S_1$ ) and second ( $S_2$ ) recurrent generations and that selection reduced the overall population variability to glyphosate. Interestingly, both the  $S_1$  and  $S_2$  populations selected for glyphosate resistance demonstrated sex ratios skewed towards maleness. Since variability for glyphosate resistance remained in the  $S_2$ , a three-level selection strategy was used to isolate asexually propagated plants with a homogenous response to glyphosate. This research suggested that resistance can evolve in a common waterhemp population with a variable response to glyphosate.

Hybridization between the glyphosate resistant horseweed and dwarf fleabane (*Conyza ramosissima* Cronq.) ranged from 0% to 9% in assisted crosses and > 95% in artificial crosses. The interspecific hybrid ( $F_1^H$ ) was phenotypically intermediate to both parents, but shared more homology to the dwarf fleabane parent. Stability, heterosis, and absence of reproductive barriers confirmed that the  $F_1^H$  was fertile. Inheritance of glyphosate resistance in the  $F_1^H$  followed the hybrid resistance model and the nuclear encoded, incompletely-dominant single gene (*R*-allele) model in the hybrid progeny ( $F_2^H$ ). We argue that adequate fitness and niche differentiation are prerequisites for successful hybrid adaptation in the environment and that hybridization in *Conyza* may complicate the containment of glyphosate resistance in current agroecosystems.

## CHAPTER 1. GENERAL INTRODUCCION

### Introduction

Glyphosate (*N*-(phosphonomethyl) glycine) is a broad-spectrum, nonselective herbicide with low mammalian toxicity and rapid soil degradation, thus minimally contaminating underground waters.<sup>(1)</sup> In plants, glyphosate inhibits 3-phosphoshikimate 1-carboxyvinyltransferase (EPSPS, EC 2.5.1.19) in the shikimate pathway<sup>(2)</sup> and blocks cytoplasmically synthesized EPSPS preprotein (pEPSPS) import into chloroplasts.<sup>(3)</sup> EPSPS catalyzes the reversible addition of the enolpyruvyl moiety of phosphoenolpyruvate (PEP) to shikimate-3-phosphate (S3P) and forms 5-enolpyruvylshikimate-3-phosphate (EPSP), which leads to the synthesis of important plant metabolites (Appendix 2).<sup>(2,4)</sup>

Few plant species have evolved glyphosate resistance despite the extensive and prolonged worldwide use of glyphosate.<sup>(5)</sup> Potential molecular mechanisms for glyphosate resistance include overexpression<sup>(6,7)</sup> or amplification<sup>(8,9)</sup> of *EPSPS* and point mutations<sup>(10,11)</sup>, and enhanced half-life<sup>(7)</sup> or increased specific activity of EPSPS.<sup>(7,12,13)</sup> At the plant level, mechanisms for resistance could include differential glyphosate absorption<sup>(14)</sup>, translocation<sup>(15,16)</sup>, metabolism<sup>(17,18)</sup>, and sequestration in cellular compartment as vacuoles and plant structures as laticifers.<sup>(19)</sup> Evidence in field bindweed (*Convolvulus arvensis* L.) suggested that multiple mechanisms at the cellular and metabolic levels acted concomitantly to mediate glyphosate resistance.<sup>(20)</sup>

Eight pigweed species exist in the Great Plains of the United States (U.S.); control with postemergence herbicides varies depending on the specific herbicide, application timing, and pigweed species.<sup>(21,22)</sup> Management of common waterhemp [*Amaranthus tuberculatus* (Mq. ex DC) J.D. Sauer] has become problematic because of the emergence pattern<sup>(23)</sup>, potential for interspecific hybridization<sup>(24)</sup>, and evolved multiple- and cross-resistance to triazine, acetolactate synthase (ALS, EC 2.2.1.6)- and protoporphyrinogen oxidase (PPO, EC 1.3.3.4)-inhibiting herbicides.<sup>(25-27)</sup> Another weed demonstrating variable response to herbicides, and recently evolving resistance to glyphosate, is horseweed [*Conyza canadensis* (L.) Cronq.].<sup>(28)</sup> Iowa farmers have reported inconsistent common waterhemp control with glyphosate; therefore, this investigation assessed the potential for selection of

glyphosate resistance in a common waterhemp population. In addition, the introgression of glyphosate resistance gene(s) was investigated by ascertaining the potential for hybridization between horseweed and dwarf fleabane (*Conyza ramosissima* Cronq.), the latter is also indigenous to U.S. ecosystems.

### Project Justification

Glyphosate resistant crop systems allow the selective postemergence use of glyphosate, thus providing an excellent weed management strategy. However, reliance on this system has increased glyphosate use and may, therefore, promote the evolution of glyphosate resistant weeds. The short half-life in the environment, unique biochemical characteristics, and the complex molecular modifications required to engineer glyphosate resistant crops suggested that the evolution of glyphosate resistant weeds was improbable.<sup>(5,29)</sup> Nevertheless, species inter- and intra-genetic variability may lead to weed population shifts and select for individuals with an enhanced fitness for glyphosate.<sup>(30)</sup> Determining the potential for glyphosate resistance to evolve may assist in preventing future weed management problems.

U.S. farmers harvested a half a million ha of Roundup Ready<sup>®</sup> soybeans (*Glycine max* (L.) Merr.) in 1996 representing 2% of the total soybean production; by 1999 this area increased to 14 million ha.<sup>(31)</sup> Currently, glyphosate resistant soybean and cotton (*Gossypium hirsutum* L.) varieties predominate the area planted in the U.S. However, limited information exists regarding the implications of adopting this technology at a nationwide scale. The available information describes mammalian toxicology experiments<sup>(32-35)</sup>, the development of tolerance in crops<sup>(36-38)</sup>, crop performance in the field<sup>(39,40)</sup>, and the characterization of the transgenic *EPSPS* expression.<sup>(41,42)</sup> Negligible information exists about the impact(s) of glyphosate resistant crops on the weed community. Recently, environmental organizations have warned about risks associated with glyphosate resistant crops to the environment, human health, and the farm economy.<sup>(43)</sup> These perils include concerns for the appearance of new allergens, lower nutritional value of agricultural products, the release of genes without evolutionary selection, and a reduction of international grain prices.<sup>(43,44)</sup> Therefore, this investigation evaluated the sustainability of glyphosate resistant crops by assessing the

potential for evolution of glyphosate resistance in common waterhemp and the introgression of resistance gene(s) in two *Conyza* species. Additionally, a genetic model for the inheritance of resistance gene(s) is proposed.

### **Dissertation Organization**

This manuscript is divided into four chapters and one addendum: the first chapter contains a comprehensive literature review, including an overview of the shikimic acid pathway, the topology and kinetics of EPSPS, the structure and evolution of *EPSPS*, and a description of glyphosate and its loci of action, two chapters follow that chronicle the actual research, and a final general conclusions and recommendations chapter. The addendum describes research conducted to characterize the evolved resistance to acetolactate synthase-inhibiting herbicides in Iowa populations of common sunflower (*Helianthus annuus* L.), giant ragweed (*Ambrosia trifida* L.), and shattercane (*Sorghum bicolor* (L.) Moench).

Chapter two in this dissertation describes the recurrent selection of common waterhemp from Everly, Iowa, the characterization of glyphosate resistant and susceptible populations, and the description of an asexual propagation method to perpetuate genotypes of interest. The subsequent chapter describes the potential for hybridization between horseweed and dwarf fleabane, estimates of postzygotic reproductive barriers for the interspecific hybrids, and proposes a genetic model for the inheritance of gene(s) associated with glyphosate resistance. References cited within each section are presented at the end of each chapter.

### **Literature Review**

#### **Overview of the shikimic acid pathway**

Most plants and microorganisms are autotrophic and thus grow adequately with sufficient environmental resources. Conversely, animals synthesize only half of the compounds essential for life and thus rely on autotrophic organisms to acquire the rest, including the aromatic amino acids L-phenylalanine, L-tyrosine, and L-tryptophan synthesized through the shikimate pathway.<sup>(4)</sup> PEP from glycolysis and erythrose-4-phosphate (E4P) from the pentose phosphate pathway are converted to chorismate in the



shikimate pathway, which in turn, serves as a precursor for the synthesis of the aromatic amino acids, alkaloids, anthocyanins, auxins, coumarins, flavonoids, folates, lignins, phenylpropanoids, quinones, and tannins (Appendix 2).<sup>(2,4)</sup> Roughly 40% of the carbon fixed by plants can be routed through the shikimate pathway<sup>(45,46)</sup> accounting for up to 60% of the total plant dry weight.<sup>(4)</sup> Regulation of the shikimate pathway is thought to occur at the level of 2-keto-3-deoxy-D-arabinoheptulosonate-7-phosphate synthase (DAHPS, EC 4.1.2.15), by feedback inhibition of the aromatic amino acids.<sup>(47)</sup> Despite the importance of the shikimate pathway, little is known of the regulation and allocation of metabolites leading to the synthesis of aromatic compounds.

### **3-phosphoshikimate 1-carboxyvinyltransferase (EPSPS)**

#### *Kinetics of EPSPS*

Preliminary enzyme isolation and elucidation of EPSPS kinetics were performed in *Escherichia coli*.<sup>(48)</sup> The reversible addition-elimination model purported a two-stage reaction, initiated with a nucleophilic attack of the C<sub>5</sub>-OH of S3P on the C<sub>β</sub> of PEP, thus facilitating protonation of the C<sub>γ</sub> and forming a tetrahedral intermediate.<sup>(49)</sup> Furthermore, a base in EPSPS instigated rearrangements of the vinylic protons and donated the unshared pair of electrons to the phosphoryl group in the tetrahedral moiety (Appendix 3). The second stage comprised the oxidation of the ester bond, regeneration of the base in EPSPS, and the release of EPSP plus inorganic phosphate (P<sub>i</sub>).<sup>(50,51)</sup> The mechanism of EPSPS is rare in nature as the enolpyruvyl moiety of PEP is transferred regiospecific and unmodified in a reversible addition-elimination model.<sup>(4)</sup> Generally, most PEP-utilizing enzymes cleave the phosphorus-oxygen and not the carbon-oxygen bond of PEP.<sup>(49)</sup>

While an equilibrium sequential ordered mechanism was initially proposed<sup>(52)</sup>, further evidence demonstrated that EPSPS follows a random reversible kinetic mechanism<sup>(53-55)</sup>, reacting through a tightly-bound kinetically competent EPSPS·S3P·PEP tetrahedral intermediate.<sup>(50)</sup> The single noncovalent tetrahedral intermediate mechanism for EPSPS was challenged by solid-state nuclear magnetic resonance (NMR) spectroscopy analysis that purported a two covalent EPSPS-intermediates mechanism.<sup>(56)</sup> Nevertheless, this allegation was recently refuted by data demonstrating that the reported species were the product of

EPSP and a side-product EPSP ketal.<sup>(57,58)</sup> In addition, the stable tetrahedral intermediate concept has been reaffirmed by kinetic competence<sup>(59)</sup> and pulsed-flow electrospray mass spectrometry measurements.<sup>(60)</sup>

### *Topology of EPSPS*

The first attempt at elucidating the crystal structure of EPSPS produced hexagonal bipyramid crystals suitable only for medium-resolution single-crystal X-ray diffraction studies at 3.8 Å.<sup>(61)</sup> A more recent electron density map at 3 Å resolution revealed that the EPSPS monomer consists of two globular domains, each of approximately 50 Å in diameter and linked by two crossover chain segments.<sup>(62)</sup> Each domain has a distinctive three-fold symmetry axis defined by three folding units of two parallel  $\alpha$ -helices and four-stranded  $\beta$ -sheets; both the amino and carboxyl termini reside in the lower domain. The principal secondary structure consists of three parallel  $\alpha$ -helices buried by a hydrophilic surface which is formed from three superficial  $\beta$ -sheets and three parallel  $\alpha$ -helices. Topological analysis identified a flat surface at the crossover of both domains, defined by the amino terminus of 12 coalescent  $\alpha$ -helices, which formed a "V" structure.<sup>(62)</sup> Substrates, inhibitors, and the tetrahedral intermediate of EPSPS possess multiple-charged anions. Therefore, the perpendicular orientation of both planes created a helical macro-dipole effect with maximal charge density and a positive gradient field at the center of the enzyme, thus guiding ligands into the active site near the crossover region of both domains.<sup>(63)</sup> More recently, a crystal-structure analysis at 1.5 Å clarified at atomic detail the interactions of EPSPS with S3P and glyphosate.<sup>(64)</sup>

### *Structure and evolution of EPSPS*

In bacteria, EPSPS is encoded by the cistron *aroA*.<sup>(65)</sup> In contrast, algae, fungi, and cyanobacteria possess the *arom* polycistron that encodes a pentafunctional enzyme catalyzing the five sequential reactions from DAHP to EPSP in the shikimate pathway; EPSPS is encoded by *aroA* in the *arom* polycistron.<sup>(66-68)</sup> The nuclear *EPSPS* of higher plants codes for a monomeric-monofunctional peptide of approximately 427 amino acids and 46112 molecular mass ( $M_r$ ).<sup>(69-71)</sup> Plant EPSPS is predominantly functional in plastids.<sup>(72)</sup> pEPSPS

is synthesized by free cytoplasmic ribosomes and imported into the plastid through recognition of an amino-terminal transit peptide sequence ( $_N$ TPS); proteolytic cleavage of  $_N$ TPS yields the mature 48 kDa enzyme.<sup>(73)</sup> In petunia (*Petunia hybrida*), *EPSPS* has a 9 kb open reading frame (ORF) with seven introns; upon translation, the initial 72 amino acids comprise the  $_N$ TPS, while lysyl<sup>73</sup> defines the initial residue of the mature enzyme.<sup>(74,75)</sup> Similar gene structures were reported in arabidopsis (*Arabidopsis thaliana* (L.) Heynh.), tomato (*Lycopersicon esculentum* L.), and canola (*Brassica napus* L.), however, introns were significantly smaller.<sup>(6,75,76)</sup> Nucleotide comparisons reported 23% to 58% and 83% to 93% homologies between the  $_N$ TPS and *EPSPS* of petunia, arabidopsis, tomato, and tobacco (*Nicotiana tabacum* L.), respectively.<sup>(6,75,77)</sup> Extensive conservation of nucleotide regions was reported between plant, bacteria, and fungi, thus suggesting that *EPSPS* has been stable through evolution.<sup>(78)</sup> Nevertheless, phylogenetic analyses indicated that plant and bacteria *EPSPS* separated later in evolution, compared to the divergence between plant and fungi *EPSPS*.<sup>(75)</sup> As an endosymbion, plant *EPSPS* probably migrated from the plastid into the nucleus and thus evolved from the original prokaryotic gene.<sup>(76,79)</sup> The fact that bacterial *EPSPS* does not fit the model of sequentially repeated folding units in a simple symmetric molecule suggested that the *EPSPS* evolved from replication of a primordial folding unit.<sup>(62)</sup>

#### *Expression of EPSPS homologs*

Petunia *EPSPS* mRNA levels are developmentally regulated and accumulate primarily in petals.<sup>(80)</sup> Petal-specific expression requires an -1800 to -800 bp 5'-upstream sequence from *EPSPS* and -1390 to -1143 bp for root cortex-, trichome-, and stem meristematic-specific expression in seedling; this extensive sequence requirement (>245 bp) indicated that several redundant and distant *cis*-elements control the tissue-specific expression of *EPSPS* in plants.<sup>(80)</sup> Concomitantly, DNase I footprinting assays characterized four strong and several weak promoter sites that appeared to bind to the single *trans*-acting factor EPF1.<sup>(81)</sup> EPF1 contained an  $\alpha$ -helix with two Cys<sub>2</sub>-His<sub>2</sub> zinc finger motifs for putative interaction with the major groove of B-DNA. Further work cloned additional novel genes encoding zinc finger proteins with binding specificity similar to EPF1.<sup>(82)</sup> In maize (*Zea mays* L.) and sorghum (*Sorghum bicolor* (L.) Moench.), two *EPSPS* homologs have

been characterized with similar structural, kinetic, and biochemical properties; however, one isoform was constitutively expressed while the second demonstrated developmental regulation.<sup>(83,84)</sup> The differential expression of *EPSPS* homologs provided support to the dual-pathway hypothesis for aromatic compound production, purporting that sink tissues contain an *EPSPS* for synthesis of aromatic amino acids and precursors of lignification, while *EPSPS* in source tissues supports aromatic amino acid production for leaf protein synthesis or export to sink organs.<sup>(85,86)</sup>

### **Loci of glyphosate action**

One mechanism proposed that glyphosate inhibits *pEPSPS* import into chloroplasts<sup>(3)</sup>; however, the accepted mechanism is the competitive inhibition of *EPSPS* with respect to the phosphate moiety in *PEP*.<sup>(2,87)</sup> Isothermal titration microcalorimetry,<sup>(53)</sup> equilibrium fluorescence,<sup>(88)</sup> and NMR spectroscopy<sup>(89-91)</sup> determinations confirmed that inhibition occurred through an intermediate-stable ternary *EPSPS*·*S3P*·glyphosate complex. Thus, a forward equilibrium-ordered mechanism was proposed, suggesting that glyphosate behaved as a transition-state analog of *PEP* in the ternary complex.<sup>(52,92,93)</sup> The proposed transition-state analog model was discredited by recent studies demonstrating a random addition mechanism for *EPSPS*<sup>(54,55)</sup> and the formation of a quaternary *EPSPS*·*EPSP*·glyphosate-*P<sub>i</sub>* complex.<sup>(94)</sup> The spatial orientation of glyphosate within either the ternary or quaternary complexes is unknown, however NMR studies have suggested that the carboxylate carbon in glyphosate is 7.2 Å from the phosphate group of *S3P*.<sup>(90)</sup> This implies that both glyphosate and *S3P* are relatively close in the ternary complex and that glyphosate probably does not bind directly over the active site of *EPSPS*.<sup>(94,95)</sup> Elucidation at the atomic detail confirmed that the glyphosate phosphate group hydrogen bonds with glutamyl<sup>171</sup>, arginyl<sup>124</sup>, glycyl<sup>96</sup>, and asparagyl<sup>94</sup>, the amino group with glutamyl<sup>341</sup>, and the carboxylate carbon with arginyl<sup>344</sup> and arginyl<sup>386</sup>. Furthermore, adherence of glyphosate and *S3P* to *EPSPS* requires a water molecule that binds the phosphate groups in both substrates of the enzyme by hydrogen bonds between the amino and C<sub>5</sub>-OH groups.<sup>(64)</sup>

## Glyphosate resistance at the cellular and molecular levels

### *EPSPS amplification*

The earliest report confirmed an eight-fold resistance increase to glyphosate in *E. coli* cells overexpressing EPSPS by amplification of the *aroA* plasmid.<sup>(65)</sup> Petunia cells acclimatized to increasing glyphosate concentrations demonstrated an enhanced enzyme activity resulting from a 20-fold increase in endogenous glyphosate-sensitive EPSPS<sup>(71)</sup> and elevated *EPSPS* mRNA levels.<sup>(74)</sup> In glyphosate-resistant tobacco cells, the level of resistance correlated with the increase in EPSPS specific activity and the amplification of at least two *EPSPS* homologs.<sup>(96)</sup> Growth in a glyphosate-free medium for eight months maintained *EPSPS* amplification levels comparable to the original glyphosate-resistant tobacco cells, suggesting that the amplification of *EPSPS* was a stable genetic modification.<sup>(77)</sup> While the level of *EPSPS* amplification prevailed even after a three year growth in glyphosate-free medium, plantlets from these cells displayed a 20-fold decline in resistance compared to plantlets regenerated from cells grown under continuous glyphosate selection. Reports with glyphosate resistant carrot (*Daucus carota* L.) cells are consistent with those for tobacco cells, except that EPSPS activity gradually declined until the fifth year of selection in a glyphosate-free medium.<sup>(97,98)</sup>

### *EPSPS point mutations*

A single cytosine<sup>1703</sup> to thymine transition resulted in a prolyl<sup>101</sup> to seryl substitution in the bacterial *aroA* allele conferring a nine-fold IC<sub>50</sub> increase for glyphosate; interestingly, the prolyl<sup>101</sup> mutation disturbed the ligand binding dynamic of PEP and S3P and resulted in a kinetically inefficient EPSPS.<sup>(99)</sup> Since studies have confirmed that prolyl<sup>101</sup> is unlikely involved in catalysis of EPSPS but residues in the amino terminus of a buried  $\alpha$ -helix near the putative active site of the enzyme, mutation at this position could perturb adjacent peptidyl residues and influence the dynamics of EPSPS ligand binding interactions.<sup>(62)</sup> Another point mutation, glycyl<sup>96</sup> to alanyl, increased EPSPS IC<sub>50</sub> for glyphosate by 500-fold, however the enzyme demonstrated a 72-fold reduction in specific activity compared to the wild-type EPSPS.<sup>(11,100)</sup> Comparable results were reported for this point mutation in bacteria and algae.<sup>(68,101)</sup> Double alanyl<sup>96</sup> and seryl<sup>101</sup> point mutations magnified the level of

glyphosate resistance compared to the single alanyl<sup>96</sup> mutation, suggesting that these two positions impair glyphosate binding at distinct regions of EPSPS.<sup>(11)</sup> Other double mutations that increased EPSPS  $K_M$  for substrates and  $K_i$  for glyphosate are the glycyl<sup>101</sup> to alanyl plus glycyl<sup>144</sup> to aspartyl and glycyl<sup>101</sup> to alanyl plus prolyl<sup>167</sup> to seryl.<sup>(102)</sup>

#### *Enhanced EPSPS transcription and half-life*

Pink corydalis (*Corydalis sempervirens* (L.) Pers.) cells evolved resistance after a five months selection in 5 mM glyphosate media.<sup>(103)</sup> Resistant cells demonstrated a 10- to 30-fold EPSPS activity increase of the endogenous glyphosate-sensitive enzyme.<sup>(70)</sup> The fact that glyphosate resistance was not associated with gene amplification, suggested that either transcriptional or post-transcriptional mechanisms enhanced the expression of *EPSPS*.<sup>(104)</sup> Run-off transcription assays confirmed that overexpression resulted from higher transcription rates compared to susceptible cell cultures.<sup>(7)</sup> In an analogous approach, petunia *EPSPS* was fused to the cauliflower mosaic virus 35S promoter and introduced into arabidopsis.<sup>(74)</sup> Northern blot analysis indicated that the 35S promoter enhanced *EPSPS* expression by 20-fold compared to the endogenous promoter in arabidopsis, thus conferring glyphosate resistance in the transformed calli.<sup>(6)</sup> Whereas glyphosate resistant pink corydalis cells demonstrated elevated *EPSPS* expression, in the presence of glyphosate, EPSPS half-life doubled and lead to further increase in specific activity.<sup>(7)</sup> Therefore, at least two synergistic mechanics may control the level of glyphosate resistance in plants.<sup>(104)</sup>

#### **Glyphosate resistance at the whole-plant level**

##### *Reduced glyphosate absorption/translocation*

Limited glyphosate translocation to the roots may lead to inconsistent control in perennial weeds<sup>(105,106)</sup>, thus reduced translocation mechanisms have been associated with tolerance of quackgrass (*Elytrigia repens* (L.) Nevski) and alligatorweed (*Alternanthera philoxeroides* (Mart.) Griseb.) to glyphosate.<sup>(15,16)</sup> Additionally, reduced glyphosate influx into cells could be mediated by a modified phosphate transporter in the plasma membrane.<sup>(14)</sup> Furthermore, glyphosate sequestration may occur in laticifers, as in leafy spurge (*Euphorbia esula* L.).<sup>(19)</sup> Laticifers are thought to function at least partially in the sequestration of

xenobiotics and this mechanism may explain the limited control of laticifer-containing weeds by some herbicides.<sup>(19,107)</sup>

### *Enhanced glyphosate metabolism*

The main glyphosate metabolic pathway involves the oxidative cleavage of the C $_{\alpha}$ -N bond to yield aminomethylphosphonic acid (AMPA) and glyoxylate.<sup>(108)</sup> An alternative pathway to sarcosine was proposed that involved rupture of the C $_{\beta}$ -P bond by a novel lyase; sarcosine was further metabolized to glycine by sarcosine oxidase (EC 1.5.3.1) or sarcosine dehydrogenase (EC 1.5.99.1).<sup>(109)</sup> Most plants demonstrate a modicum of glyphosate metabolism, yet 10% to 20% of the total [ $^{14}\text{C}$ ]-glyphosate was recovered as  $^{14}\text{CO}_2$  in leaves of tobacco and sugar beet (*Beta vulgaris* L.).<sup>(110)</sup> While significant differences were reported within wheat (*Triticum aestivum* L.), soybean, and maize cell extracts, metabolism to AMPA represented 30% of the total [ $^{14}\text{C}_{\beta}$ ]-glyphosate applied in soybeans.<sup>(17)</sup>

Three glyphosate metabolizing genes, *igrA*, *glpA*, and *hph*, have been isolated from bacteria. In *Pseudomonas* sp. PG2982, the *igrA* ORF coded for a  $M_r$  39,396 protein that conferred resistance to glyphosate when engineered into *E. coli*.<sup>(111)</sup> The *igrA* protein is thought to metabolize glyphosate by oxidation of the C $_{\alpha}$ -N bond and is currently classified as member of the aldo-keto reductase superfamily.<sup>(112)</sup> In another similar study, *E. coli* transformed with a 3.0 kb DNA plasmid used glyphosate as sole phosphorous source.<sup>(113)</sup> Nucleotide sequence analysis of the plasmid identified the *glpA* and *hph* ORFs; *glpA* was homologous to hygromycin phosphotransferase which encodes an antibiotic that precludes protein synthesis. Tobacco transformation with *hph* led to resistance to 2.0 mM glyphosate in cells and 0.24 kg ae ha $^{-1}$  in regenerated plantlets.<sup>(114)</sup> Recently, glyphosate oxidase reductase (*GOX*) was isolated from *Achromobacter* sp. stain LBAA which oxidized glyphosate to AMPA; concomitant engineering of *CP4-EPSPS* and *GOX* instigated high levels of glyphosate resistance in wheat<sup>(115)</sup> and sugar beet.<sup>(38)</sup>

### **Summary**

Glyphosate represents an important weed management tool in current crop production systems. However, over-reliance on this chemistry may lead to weed resistance and

diminish the importance of this herbicide. Farmers typically base weed management decisions on the economic return and simplicity of the control tactic(s). However, management practices that favor the evolution of herbicide resistance in weed populations should be combined with alternative weed control tactics, thus maintaining the efficacy of our current weed control programs and preserving the sustainability of agricultural ecosystems.

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## CHAPTER 2. DIFFERENTIAL RESPONSE OF *AMARANTHUS TUBERCULATUS* (MQ. EX DC) J.D. SAUER TO GLYPHOSATE IN IOWA<sup>1</sup>

A paper to be submitted to *Pest Management Science*<sup>2</sup>

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### Abstract

Iowa farmers have reported inconsistent control of common waterhemp [*Amaranthus tuberculatus* (= *rudis*) (Mq.ex DC) J.D. Sauer] with glyphosate in glyphosate resistant crops. Considerable variation in glyphosate efficacy was observed within *A. tuberculatus* plants grown from seed collected near Everly, Iowa. Whole-plant rate responses verified that the Everly population had a GR<sub>50</sub> of 0.62 kg ha<sup>-1</sup> compared to 0.24 kg ha<sup>-1</sup> glyphosate of a pristine *A. tuberculatus* population from Paint Creek, Ohio. Based on these data, a divergent recurrent selection was implemented to isolate the most resistant and susceptible genotypes within the Everly population. A seedling assay was developed to screen large amounts of seeds and thus expedite the recurrent selection process. The first recurrent generation selected at 3.2 mM glyphosate for resistance (S<sub>1</sub>-R) and 32 µM glyphosate for sensitivity (S<sub>1</sub>-S) had a 5.9 fold resistance increase at the seedling and a 1.7 fold resistance at the whole-plant level. The second recurrent generation selected at 8 mM (S<sub>2</sub>-R) and 10 µM (S<sub>2</sub>-S) glyphosate had a 3 fold resistance increase at the whole-plant level. Recurrent selection increased the level of resistance and decreased the variability to glyphosate at the population

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level. Interestingly, populations selected for resistance to glyphosate were comprised of predominantly male plants, which suggested that resistance and plant sex may be associated traits in *A. tuberculatus*. Significant variability for glyphosate resistance was observed even after two cycles of recurrent selection. Therefore, a three-level selection strategy was implemented to confirm that resistance in the seedling level was expressed in mature plants. Selected plants were reproduced asexually (cloned) and individually characterized based on the pattern of endogenous shikimic acid accumulation in response to 0.86 kg ha<sup>-1</sup> glyphosate. Results herein reported suggest that *A. tuberculatus* is inherently variable to glyphosate and that selection increases the frequency of resistant individuals with the population. Since selection pressure under field conditions depends on several biotic and abiotic factors, we purport that glyphosate resistance in *A. tuberculatus* may require many cycles of selection to evolve.

### Introduction

Glyphosate (*N*-(phosphonomethyl) glycine) resistance was first engineered in soybeans (*Glycine max* L. Merr) through particle acceleration transformation of *CP4 EPSPS*; fusion to a chloroplast transit nucleotide sequence translated into high resistance levels *in planta*, while preserving feed quality and exhibiting no perceivable yield penalty in the transformed crop.<sup>1</sup> Since the commercialization in 1996, glyphosate resistant soybeans have predominated markets covering in 1999 at least 14 million hectares of the crop land planted in the United States (U.S.).<sup>2</sup> Glyphosate use in resistant crop systems provides excellent control of a broad weed spectrum, thus simplifying weed management, presumably lowering production costs, and permitting the management of weeds resistant to other herbicides.<sup>3,4</sup> While glyphosate resistant crops may provide a nutritional value, grain yield, and exhibit a level of pathogen resistance comparable to conventional crops, under some conditions glyphosate resistant crops may yield lower than their non-transgenic counterparts.<sup>5-10</sup> For example, high temperatures can increase glyphosate translocation into the meristems, thus adversely affecting leaf chlorophyll content, symbiotic nitrogen fixation, and plant growth.<sup>11-14</sup> Moreover, glyphosate resistant crops are typically treated with only glyphosate which increases the selection pressure and promotes the evolution of resistant weed populations.<sup>3,4</sup>

Glyphosate tolerance is well documented in several plant species.<sup>15-19</sup> Resistance was first confirmed in two independent rigid ryegrass (*Lolium rigidum* Gaudin) populations where 7- to 11-fold resistance evolved after 15 years of continuous glyphosate applications.<sup>20,21</sup> Differences in 3-phosphoshikimate 1-carboxyvinyltransferase (EPSPS, EC 2.5.1.19) kinetics, *EPSPS* mRNA levels, and uptake, translocation, or metabolism of glyphosate failed to account for the response observed *in planta*, thus suggesting that resistance in *L. rigidum* may be conferred by improper glyphosate targeting to the loci of action.<sup>22,23</sup> Regardless, glyphosate resistance in *L. rigidum* was apparently conferred by a single nuclear gene inherited in an incomplete-dominant fashion.<sup>24</sup> In a related species, Italian ryegrass (*Lolium multiflorum* Lam.), 4- to 6-fold resistance evolved after 8 to 10 years of continuous glyphosate use; the resistance mechanism is to date unknown.<sup>25</sup> Goosegrass (*Eleusine indica* (L.) Gaertn.) evolved 8- to 12-fold resistance after only four years of glyphosate application; but contrary to *L. rigidum* and *L. multiflorum*, the mechanism was ascribed to a polymorphic EPSPS with high dissociation constant ( $K_i$ ) for glyphosate derived from a prolyl<sup>106</sup> to seryl point mutation at the enzyme's putative active site.<sup>26,27</sup> Furthermore, glyphosate resistance has evolved independently in several horseweed (*Conyza canadensis* (L.) Cronq.) populations within the U.S.<sup>28</sup> *C. canadensis* differs from other species in that resistant plants demonstrate injury and accumulate shikimic acid transiently until several days after glyphosate application.<sup>29</sup> While no clear mechanism has been elucidated to date, an investigation in field bindweed (*Convolvulus arvensis* L.) suggested that several mechanisms at the cellular and metabolic levels may act concomitantly to affect glyphosate resistance.<sup>18</sup>

The cosmopolite pigweed complex is represented by at least eight weedy species in the Great Plains of the U.S.<sup>30</sup> Control with postemergence herbicides is variable depending on the pigweed species and the application timing.<sup>31,32</sup> In recent years however, multiple-resistance to triazine, acetolactate synthase (ALS, EC 2.2.1.6) and protoporphyrinogen oxidase (PPO, EC 1.3.3.4) inhibiting herbicides has complicated common waterhemp (*Amaranthus tuberculatus* (= *rudis*) (Mq. ex DC) J.D. Sauer) management, even with alternating herbicide programs.<sup>33-35</sup> Recently, multiple incidents of inconsistent *A. tuberculatus* control with glyphosate in glyphosate resistant crops were reported by Iowa

farmers. In Everly IA, a farmer claimed that rates of 0.63 kg acid equivalents (ae) isopropylamine salt of glyphosate ha<sup>-1</sup> on 10 cm tall plants and followed by 0.83 and 1.25 kg ha<sup>-1</sup> one and two weeks after the initial glyphosate application failed to control *A. tuberculatus* effectively. This incident occurred during the first year of glyphosate-resistant soybeans (*Glycine max* L. Merr) use by the farmer. Preliminary greenhouse assessments of the Everly, IA material suggested that plants originated from individuals with a resistant phenotype demonstrated an enhanced fitness to glyphosate compared to an unselected *A. tuberculatus* population. Subsequently, an investigation was undertaken to assess the potential for selection of glyphosate resistance in the Everly, IA *A. tuberculatus* population.

## Materials and Methods

### Plant materials, reproduction, and seed conditioning

Ninety-seven plants (53 ♀: 44 ♂) with a resistant phenotype were collected from the Everly, Iowa field where glyphosate allegedly failed to control *A. tuberculatus*. Plants were transplanted to pots containing a peat:perlite:loam soil-mix (1:2:1), irrigated as needed, and fertilized (Miracle Gro Excel, Scott-Sierra Co. 14111 Scottlawn Road. Marysville, OH. 43041) twice during the reproductive growth cycle. Natural light was supplemented to 16 hrs with artificial illumination delivering 600 to 1000  $\mu\text{mol}^{-2} \text{m s}^{-1}$  photosynthetic photon flux density (PPFD). Greenhouse conditions were maintained at 25 to 30 C and 40 to 60% relative humidity (RH) diurnal and 20 to 25 C and 50% RH nocturnal conditions. At anthesis plants, were permitted to cross without pollination restrictions. When fully mature, seeds from individual female plants (maternal lines) were collected in DelNet<sup>®</sup> bags (Speciality Nets & Profiles. 601 Industrial Drive. Middletown, DE. 19709), cleaned in an air column separator, and stored at 5 C and darkness until used. This seed comprised the parental Everly, IA population (P<sub>0</sub>-E). The wild type *A. tuberculatus* populations (P<sub>0</sub>-W) collected at Paint Creek Dam, OH (PI 603888) was obtained through the USDA-ARS National Plant Germplasm System (NPGS). Dormancy was overcome by conditioning seeds in filter paper moistened (20 mL) with a 1 mM GA<sub>3</sub> (90% (+)-gibberellic acid) solution in plastic petri dishes. Finally, plastic petri dishes were placed at 5 C and dark conditions for one month.

### Seedling dose response

Each of the 53 maternal lines were evaluated for response to glyphosate utilizing a seedling assay. For each line, twenty seeds per well in a 24 well cell culture cluster plate (Corning Incorporated. South Bank Chemung River. Corning, NY. 14831) were placed on four 3 mm diameter 3MM chromatography paper discs (Whatman Inc. 100 Ames Pond Drive. Tewksbury, MA. 01876-0962) previously moistened (500  $\mu$ L) with distilled-deionized water ( $\text{ddH}_2\text{O}$ ) or doses of 32 mM, 10 mM, 3.2 mM, 1 mM, 0.32 mM, 0.1mM, 0.032 mM glyphosate. The salt of glyphosate (Sigma-Aldrich Corporation. 3050 Spruce Street, St. Louis, MO. 63103) was solubilized in  $\text{ddH}_2\text{O}$  by adjusting pH to 7.0 with 0.5 M KOH. Three replications of the  $\text{ddH}_2\text{O}$  (control) and the glyphosate doses were established per culture cluster plate; evaporation of the treatments was prevented by enveloping plates in plastic wrap film. Plates were also established with 0.5 M KOH alone to assess the effect of the base on seed germination and seedling development. Finally, plates were placed in 30 C and 14 hrs light and 20 C and 10 hrs dark conditions at  $500 \mu\text{mol}^{-2} \text{m s}^{-1}$  PPFD. Seedling germination, hypocotyl, and radicle length were recorded two weeks after plate establishment. Germination was defined as radicle protrusion from the seed; length measurements were recorded from the hypocotyl base to the seedling apex or root distal end.

### Whole-plant rate response

Seeds were planted in a 30 x 90 cm flat with soil-mix and one week after emergence, three seedlings per 12 cm diameter pot were transplanted equidistantly and grown under the conditions previously disclosed. The manufacturer's recommended rate for 15 cm tall *A. tuberculatus* was  $0.85 \text{ kg ha}^{-1}$  glyphosate, thus rate responses were conducted at 0.0 ( $\text{ddH}_2\text{O}$ ), 0.21, 0.54, 0.85, 1.27, 1.69, and  $2.5 \text{ kg ha}^{-1}$ .<sup>36</sup> Treatments were sprayed in a  $\text{CO}_2$ -powered spray chamber (Model SB5-66, DeVries Manufacturing, Route 1 Box 184, Hollandale, MN 56045) 30 cm from the plant canopy using an even flat flow nozzle that delivered  $187 \text{ L ha}^{-1}$  at  $2.8 \text{ kg per cm}^2$ . Visual glyphosate injury was assessed 14 days after application by estimating the percent foliage damage of treated plants compared to the  $\text{ddH}_2\text{O}$ -treated controls; dead plants received a 100% estimate while asymptomatic plants were scored 0%. Typical injury symptoms include stunting, leaf chlorosis, and meristems necrosis. Plant



height was estimated by measuring the distance from the soil surface to the base of the apex. Plants were cut at the soil surface, the tissue dried at 35 C for 48 hrs, and biomass measured by weighing individual samples. Finally, the three dry apices per pot were pooled for shikimic acid determination (explained below).

### **Divergent recurrent selection of the Everly, IA population**

Based on the seedling dose responses, maternal lines were selected for three generations of recurrent selection utilizing the seedling assay in a divergent strategy, thus isolating glyphosate resistant (R) and susceptible (S) individuals within the populations (Table 1). Previous determinations indicated that 100 *A. tuberculatus* seeds weigh approximately 20.8 mg. Two 3.5 cm diameter chromatography paper disks were moistened with 1 ml of glyphosate solution and approximately 500 seed per well were established in a 6 well cell culture cluster. Plates were treated as previously indicated, except that seedlings were grown at  $500 \mu\text{mol m}^{-2} \text{s}^{-1}$  PPFD and pots covered with plastic bags to maintain an 80% to 90% RH. The R phenotype was classified as individuals germinating and growing in the selection media for at least two weeks; conversely, the S phenotype was comprised of individuals germinating in the media, but developing toxicity symptoms at sub-lethal glyphosate doses. Individuals that met these classifications were rescued from the selection media, the residual glyphosate rinsed in  $\text{ddH}_2\text{O}$ , and roots treated with Rootone® (TechPac, LLC, P.O. Box 24830, Lexington, KY, 40504.). Seedlings were then transplanted into an autoclaved soil-mix and grown at  $1100 \mu\text{mol m}^{-2} \text{s}^{-1}$  PPFD and 90 to 95% RH. Plants that survived these conditions were transferred to the previously described greenhouse environment. At anthesis, R or S plants were intercrossed in clear plastic tents where pollen contamination was less than 0.01%.<sup>37</sup> Finally, seeds were collected, cleaned, and conditioned as previously indicated.

### **Asexual propagation of *A. tuberculatus***

Plant propagules were prepared by defoliating *A. tuberculatus* plants, cutting at the base of main stems, and treating the abaxial end with Rootone®. The stem-cuttings were then planted in vermiculite and allowed to develop in a growth cabinet at 16 hrs of 1100

$\mu\text{mol}^{-2} \text{ m s}^{-1}$  PPFD and approximately 95% RH. Adventitious roots developed within one to three weeks after planting. Cuttings were then transplanted to an autoclaved soil-mix and fertilized weekly for one month. The development of an asexual propagation (clones) method enabled the perpetuation of specific genotypes and facilitated the increase of seed for recurrent selection. Interestingly, the long photoperiod and frequent fertilization instigated the re-differentiation of floral buds in *A. tuberculatus* back to vegetative meristems.

### **Shikimic acid assay**

#### *Shikimic acid extraction*

Dry apex samples (0.2 g) were ground in liquid nitrogen with a mortar and pestle and then moistened with 0.25 M HCl at a 1:20 (w:v) proportion. Shikimic acid was extracted by disrupting cells in a 2210 Branson Ultrasonicator (Branson Ultrasonic Corporation, 9999 Markham Road, P.O. Box 150, Markham, ONT. Canada L3P3J6) at 25 C for 30 min and plant debris separated by centrifugation at 20,000 g for 15 min. The supernatant was used directly for shikimic acid determination.

#### *Spectrophotometric shikimic acid determination*

Determination was conducted by mixing the supernatant extract (10  $\mu\text{L}$ ) with deionized water (240  $\mu\text{L}$ ) and an aqueous solution (250  $\mu\text{L}$ ) of 0.5% periodic acid plus 0.5% sodium *meta*-periodate (w:v). The mixture was then incubated at 37 C for 30 to 45 min, quenched with 3:2 (v:v) 1.0 M NaOH:0.056 M  $\text{Na}_2\text{SO}_3$  (500  $\mu\text{L}$ ), and absorbance detected at 382 nm in a Lambda 18 UV/Vis Spectrometer (Perkin-Elmer, 710 Bridgeport Avenue, Shelton, CT 06484). Samples were assayed in duplicate and standard curves constructed with shikimic acid (Sigma-Aldrich, 3050 Spruce Street, Saint Louis, MO 63103) at a range of 1 to 60  $\mu\text{mol ml}^{-1}$ . The shikimic acid chromophore had a molar absorptivity ( $\epsilon_{382}$ ) of  $4.76 \times 10^4 \text{ l mol}^{-1} \text{ cm}^{-1}$  at pH 11–12.

### **Non-destructive plant assay to assess glyphosate efficacy**

Branches from asexually propagated *A. tuberculatus* plants (clones) were cut at the base of stems and inserted in 13 x 100 mm culture tubes with hydro-grow<sup>®</sup> hydroponic

solution (Hummert International, P.O. Box 31606, Saint Louis, MO, 63131-0606). Stem-cuttings were sprayed with  $0.86 \text{ kg ha}^{-1}$  glyphosate, provided with hydro-grow hydroponic solution as needed, and placed under a 73% PAK shade fabric (Hummert International) with natural light supplemented to 16 hrs of  $600 \mu\text{mol}^{-2} \text{ m s}^{-1}$  PPFD. Glyphosate efficacy was estimated visually and by measuring shikimic acid accumulation in leaves of treated stem cuttings as indicated previously. The non-destructive plant assays permitted assessment of glyphosate efficacy in selected phenotypes, without compromising the survival of plants used for seed production in the selection procedure.

### Statistical analysis

Analyses were performed with the Statistical Analysis Software.<sup>38</sup> Experiments replicated in time were tested for patterns of covariance matrices that satisfied the Huynh-Feldt condition (option PRINTE), is accepted,  $F$ -statistics tested the within time effects and related interactions.<sup>39</sup> Dose and rate response experiments repeated once in time were arranged in a randomized complete block (RCB) design with four replications and analyzed by ANOVA (PROC GLM); Fisher's least significant difference (LSD;  $\alpha = 0.05$ ) test was used to separate means when ANOVA identified significant biotype effects. In addition, biomass data were used to estimate gamma ( $\hat{\gamma}$ ) and test whether sublethal glyphosate concentrations had a stimulatory effects on *A. tuberculatus* development (hormesis).<sup>40</sup> A parameterized Brain-Cousens model was adopted to estimate the glyphosate rate that reduced *A. tuberculatus* biomass accumulation by 50% ( $GR_{50}$ ) if a positive hormetic response was obtained; otherwise, data were analyzed by PROC NLIN following the log-logistic model.<sup>41,42</sup>  $P$ -values for the difference between two estimated  $GR_{50}$  values ( $|\lambda_{50}|$ ) were calculated by reparameterization of the Brain-Cousens model.<sup>40</sup> Visual injury data were transformed to a binomial format utilizing the classifications for R ( $\leq 30\%$ ) and S ( $>30\%$ ) phenotypes and the glyphosate dose that inflicted 50% mortality in the *A. tuberculatus* population ( $LD_{50}$ ) estimated by a modified Newton-Raphson algorithm (PROC PROBIT).<sup>43</sup> The overall data fit to the models was assessed graphically by the distribution of residuals, and statistically by a lack-of-fit (LOF) tests and by pseudo coefficients of determination [ $R^2_{(\text{pseudo})}$ ].<sup>40,42</sup> Furthermore, the total phenotypic variance ( $\sigma^2$ ) for the components in the

ANOVA was estimated with PROC VARCOMP using the restricted maximum likelihood (REML) method. The relationship strength between endogenous shikimic acid and the measured plant response parameters was estimated by Spearman's correlation analysis. Finally, a one-tailed sign test was performed to assess whether the observed sex ratios in selected populations were equally proportioned (1♀:1♂).

## Results and Discussion

### Variable *A. tuberculatus* response to glyphosate occurs in the field

Most field reports of inconsistent *A. tuberculatus* control with glyphosate are attributable to an ineffective herbicide application, sublethal herbicide dose, or delayed glyphosate application with respect to the weed's phenological stage. Visual assessment of the Everly, IA location suggested that *A. tuberculatus* plants demonstrated a variable response to the sequential applications of 0.63, 0.83, and 1.25 kg ae ha<sup>-1</sup> glyphosate. Interestingly, 1998 was the first year of glyphosate use in this field, yet different control levels were observed between adjacent plants within soybean rows. Field assessment ruled out delayed or other application problems as the probable cause for the inconsistent *A. tuberculatus* control. In 1999, another similar incident was reported in Badger, IA where 12 cm tall *A. tuberculatus* plants reportedly survived two sequential applications of 1.25 kg glyphosate ha<sup>-1</sup>. Since plants were treated at the labeled height and glyphosate dose, and no application problems were detected, the inconsistent control at Badger, IA was attributed to a differential response in the *A. tuberculatus* population. Endogenous shikimic acid accumulation estimates from putative susceptible and resistant plants in Badger, IA and from untreated plants in adjacent areas, confirmed the variable whole-plant efficacy to glyphosate observed in the field (data not shown). Similar cases of inconsistent *A. tuberculatus* control with glyphosate have been reported in other Midwest states.<sup>44</sup> These anecdotal accounts suggested that some *A. tuberculatus* populations demonstrate an inherent variable response to glyphosate.

### ***A. tuberculatus* from Everly, IA is inherently variability to glyphosate**

To minimize possible environmental, rate, and phenological factors that affect herbicide efficacy in the field, the response of the parental Everly, IA (P<sub>0</sub>-E) population to glyphosate was assessed at the seedling and whole-plant levels under controlled environments. The pristine Paint Creek, OH (P<sub>0</sub>-W) population was used as control since the *A. tuberculatus* population evolved in a non-agricultural environment without previous exposure to glyphosate. Most studies that evaluate plant performance to herbicides model the response to rates to the log-logistic equation, however this model is insensitive to discontinuous increasing or decreasing trends that may result at different herbicide rates.<sup>40</sup> The Brain-Cousens model was proposed as an alternative to the accepted log-logistic model, specifically when discontinuous response trends are observed that may distort proper estimation of the response parameters.<sup>41</sup> Therefore, the response of *A. tuberculatus* at the seedling and whole-plants levels was tested for stimulatory glyphosate effects at sublethal doses (hormesis). Seedling responses of P<sub>0</sub>-E maternal lines to glyphosate were variable; some seedlings from several lines survived 3.2 mM and 10 mM glyphosate, while others perished above the 0.1 mM concentration. Evaluation of other *A. tuberculatus* populations collected from agroecosystems in Ames, IA and Ogden, IA demonstrated similar, but lower, variable responses to glyphosate. Hence, the inherent variability in *A. tuberculatus* to glyphosate was apparently not restricted to the P<sub>0</sub>-E population.

Modeling P<sub>0</sub>-E and P<sub>0</sub>-W radicle length, plant height, and biomass data to the parameterized Brain-Cousens equation estimated  $\hat{\gamma}$  values that disregarded a putative hormetic response. Therefore, response parameters were calculated based on the log-logistic model (Table 2). The rate response of P<sub>0</sub>-E hypocotyl length data, however, resulted in a  $\hat{\gamma}$  value (38.47) that did not overlap zero at the 95% confidence interval and thus confirmed hormesis. Estimates of the model fit to the data resulted in LOF tests and  $R^2_{(\text{pseudo})}$  coefficients that confirmed the adequacy of the models to describing the response of P<sub>0</sub>-E and P<sub>0</sub>-W to glyphosate. Overall, comparisons at the seedling and whole-plant levels provided non-significant  $|\lambda_{50}|$  values suggesting that the P<sub>0</sub>-E and P<sub>0</sub>-W populations responded similarly to glyphosate (Table 2). While the P<sub>0</sub>-E population tended to germinate and survive higher glyphosate rates than P<sub>0</sub>-W, the differences were not statistically

significant (Figure 1). Nevertheless, P<sub>0</sub>-E demonstrated a greater overall  $\sigma^2$  for hypocotyl (5.88) and radicle length (2.17) in response to glyphosate compared to either parameter in P<sub>0</sub>-W ( $\sigma^2 < 0.02$ ). Greater  $\sigma^2$  in the response of P<sub>0</sub>-E to glyphosate was also observed for biomass and plant height measurements. These results indicated that while P<sub>0</sub>-E and P<sub>0</sub>-W responded similarly to glyphosate, individuals within the P<sub>0</sub>-E population were more variable to glyphosate, thus confirming our visual observations of variability in the Everly, IA field.

Variable responses to glyphosate within Amaranthaceae have been documented, and *A. tuberculatus* and *A. hybridus* were found to be the most tolerant species within the family.<sup>45</sup> Reports of incomplete *A. tuberculatus* control with glyphosate in Illinois and Missouri suggested that 10% to 15% of the individuals within these populations survived 0.84 kg glyphosate ha<sup>-1</sup>, compared to less than 2% in a known glyphosate susceptible population.<sup>44</sup> Furthermore, evaluations at 0.21 kg glyphosate ha<sup>-1</sup> identified broad injury ranges of 10% to 100% within 59 *A. tuberculatus* and *A. rudis* populations in Illinois.<sup>32</sup> Collectively these reports confirm that *A. tuberculatus* in the US Mid West is inherently variable to glyphosate. More importantly, survival of *A. tuberculatus* plants with a preexisting fitness to glyphosate may increase the proportion of resistant individuals within the population and result in reduced herbicide efficacy in the field.

### **The fitness of *A. tuberculatus* to glyphosate increases with selection**

#### *The seedling assay permitted screening of a large *A. tuberculatus* population*

Considering that a variable response to glyphosate was confirmed in the P<sub>0</sub>-E population, divergent recurrent selection was implemented to ascertain whether isolation and mating of *A. tuberculatus* individuals with a confirmed resistant or susceptible phenotype changed the overall response to glyphosate at the population level. Greenhouse estimates suggested that an individual *A. tuberculatus* female plant produced more than 80,000 seeds under ideal conditions. Thus, a seedling assay was developed to cope with the prolific nature of *A. tuberculatus* and segregate individuals that demonstrated relatively high or low responses to glyphosate. The seedling assay effectively identified individuals at 10<sup>-4</sup> frequencies within the population which would have otherwise required significant greenhouse resources if selection were conducted at the whole-plant level. Since no

difference was observed between germinating *A. tuberculatus* seeds in  $\text{d}_2\text{H}_2\text{O}$  or 0.5 M KOH, survival of plants in the seedling assay was attributed to the effect of glyphosate and not to the KOH used to solubilize the herbicide (data not shown).

*The first recurrent ( $S_1$ ) generation demonstrated greater fitness to glyphosate*

Dose responses were conducted on seeds of the 53  $P_0$ -E maternal lines to characterize the inherent glyphosate response within each line (data not shown). Based on the  $P_0$ -E seedling dose responses, approximately  $3.54 \times 10^5$  seeds of 24 maternal lines considered susceptible and were selected for susceptibility at 32  $\mu\text{M}$  glyphosate. Conversely, the  $3.84 \times 10^5$  seeds of the remaining 29 maternal lines were selected for resistance at 3.2 mM glyphosate (Table 1). This selection resulted in the isolation of 376 susceptible and 159 resistant *A. tuberculatus* seedlings and thus comprised the first recurrent generation ( $S_1$ ). The  $S_1$  susceptible seedlings demonstrated chlorosis; nonetheless the development of stems and roots were visually not affected by glyphosate. Conversely,  $S_1$  seedlings surviving 3.2 mM glyphosate had green-healthy tissues with severely atrophied roots. Treatment of these stunted roots with Rootone<sup>®</sup> promoted the development of adventitious roots from stems and facilitated the rescue of resistant seedlings.  $S_1$  seedling mortality post isolation from the selection media was 86% and 92% for the susceptible and resistant populations, respectively. The 53 susceptible ( $S_1$ -S) and 13 resistant ( $S_1$ -R) plants eventually transferred to the greenhouse had sex ratios at anthesis that did not differ statistically from the 1:1 ( $\text{♀}:\text{♂}$ ) proportion (Table 1).

Evaluation of  $S_1$ -S seeds confirmed that the selected material did not develop well above 1 mM glyphosate, while radicles and hypocotyls of  $S_1$ -R seedlings grew in 3.2 mM glyphosate (Figure 2). Furthermore, less than 0.2% of  $S_1$ -R seedlings demonstrated minor or no toxicity symptoms at 10 mM glyphosate. Stimulatory responses at sublethal glyphosate concentrations were non-significant except for the radicle response of  $S_1$ -R ( $\hat{\gamma} = 20.59$ ) (Table 2). Evaluations at the seedling and whole-plant levels based on  $|\lambda_{50}|$  values indicated that the response of  $S_1$ -R and  $S_1$ -S differed only when comparing biomass as a function of increasing glyphosate rates (Table 2). This difference accounted for approximately a 1.7 fold increase in resistance to glyphosate (Figure 2). At the population level, 0.55 kg glyphosate

ha<sup>-1</sup> was required to inflict 50% mortality in the S<sub>1</sub>-S compared to 0.78 kg glyphosate ha<sup>-1</sup> in the S<sub>1</sub>-R (Table 3). Concomitantly, shikimic acid accumulation in the apex tissue of treated S<sub>1</sub> plants correlated positively with the visual herbicide ratings and negatively with plant height or biomass measurements (Table 4). Comparison between the parental and S<sub>1</sub> *A. tuberculatus* populations suggested that the recurrent selection process reduced the variability to glyphosate. For example at the seedling level, the  $\sigma^2$  of S<sub>1</sub>-R (< 0.27) and S<sub>1</sub>-S (< 1.84) was less than the hypocotyl (5.88) and radicle length (2.17) response in the P<sub>0</sub>-E.

*Second recurrent (S<sub>2</sub>) generation selected for resistance had predominantly male plants*

The second recurrent generation (S<sub>2</sub>) was selected from 2.51 x 10<sup>5</sup> seeds for susceptibility at 10  $\mu$ M glyphosate, while 1.38 x 10<sup>5</sup> seeds were selected for resistance at 10 mM glyphosate (Table 1). The 113 asymptomatic *A. tuberculatus* seedlings rescued after 10 days in 10 mM glyphosate developed injuries when transplanted to the growth chamber and 89% eventually perished. Similarly, 88% mortality was estimated in the 320 seedlings selected for susceptibility to glyphosate. At maturity the susceptible (S<sub>2</sub>-S) and resistant (S<sub>2</sub>-R) populations were comprised of 16: 21 (♀:♂) and 0: 12 (♀:♂) plants, respectively (Table 1).

Interestingly, all 12 individuals selected for resistance differentiated into male plants. These preliminary results suggested that glyphosate may differentially affect the germination of male and female *A. tuberculatus* seeds. Alternatively, it is possible that resistance to glyphosate is inherited concomitantly with maleness in *A. tuberculatus*. Limited information exists regarding the inheritance of sex in *A. tuberculatus*. Crosses between dioecious and monoecious *Amaranthaceae* provided evidence that dioeciousness was epistatic over monoeciousness. When a monoecious female parent was crossed to a male dioecious plant, the progeny exhibited a 1:1 (♀:♂) sex ratio; in the reciprocal cross however, with a dioecious female as the parent, the progeny resulted in 100% female hybrids.<sup>46</sup> This suggested that males may be heterogametic (XY) while females monogametic (XX) and that sex has a single locus with two alleles, an X and Y loci, that determined plant sex. Under this supposition, the gene(s) associated with glyphosate resistance in *A. tuberculatus* may be located in Y loci in the chromosome.



### Assess of the association between maleness and resistance to glyphosate

Ascertaining whether glyphosate instigated differential germination of *A. tuberculatus* was difficult since seeds cannot ordinarily be sexed. Therefore, the putative linkage of maleness and glyphosate resistance was tested by backcrossing S<sub>2</sub>-R individuals to S<sub>1</sub>-R females, selecting the progeny at 8 mM glyphosate, and intercrossing the selected plants to generate a new resistant lineage (BC<sub>R</sub>). Differential sex germination would have resulted in less germination of female seeds in the 8 mM glyphosate media and reduced the overall germination in BC<sub>R</sub>. Since S<sub>2</sub>-S demonstrated a normal 1:1 (♀:♂) ratio and the combined germination across rates (82%) was not statistically different ( $LSD_{\alpha, 0.05} = 6\%$ ) from BC<sub>R</sub> (81%), differential sex germination in the 8 mM glyphosate media was disregarded. To reduce BC<sub>R</sub> mortality in the growth chamber, the selected seedlings were acclimatized to gradual daily increases of irradiance from 500 to 1100  $\mu\text{mol m}^{-2} \text{s}^{-1}$  PPFD. This selection resulted in the isolation of 7: 11 (♀:♂) BC<sub>R</sub> plants which demonstrated statistically equal male and female proportions (Table 1). Hence, the putative sex-linkage to glyphosate resistance observed in the S<sub>1</sub>-R was attributed to the small sample size in the selected material. Few reports exist that cite departure from the 1:1 (♀:♂) distribution in natural *A. tuberculatus* populations. In isolated native Ohio *A. tuberculatus* populations, female plants can comprised up to 90% of the individuals in a population.<sup>47</sup> Conversely, a tendency for more *A. tuberculatus* males was reported in response to composted swine manure.<sup>48</sup> Spatial segregation of sexes across environmental gradients is well documented in dioecious plant species, such that increased competition and stressful conditions may favor the prevalence of males in a particular time and space.<sup>49,50</sup> In tidalmarsh amaranth (*Amaranthus cannabinus* (L.) Sauer) no spatial segregation of sexes was observed due to salt stress.<sup>50</sup> However, temporal abnormal sex ratios observed under control conditions were attributed to flowering phenology differences and sex-specific mortality. This suggested that differences in gender growth pattern may be related to the evolution of dioecy in Amaranthaceae. While the distribution of sex ratios in *A. tuberculatus* populations has not been investigated extensively, the report from Ohio suggested that sex disparity is a probable but rare event.<sup>47</sup>

### Evident variability to glyphosate even after two cycles of recurrent selection

Consistent with the  $S_1$  response,  $S_2$ -S seedlings did not effectively grow above 1 mM glyphosate (Figure 3). Conversely, more radicle and hypocotyl growth was observed at 3.2 mM glyphosate in the  $S_2$ -R compared to the  $S_1$ -R (Figure 2; Figure 3). Estimates of hormesis were non-significant for the radicle, hypocotyl, biomass, and plant height data ( $\hat{\gamma} > 0.23$ ), therefore the log-logistic model was used to estimate fitness to glyphosate. Comparison based on  $|\lambda_{50}|$  values estimated a 3-fold and 14-fold resistance increase between  $S_2$ -R and  $S_2$ -S for the hypocotyl length and biomass responses, respectively (Table 2). Concomitantly, a 2.6-fold resistance increase was estimated when comparing mortality as a function of glyphosate concentration at the population level (Table 3). In addition, endogenous shikimic acid correlated positively with visual injury and negatively with plant height or biomass measurements (Table 4). Both  $S_1$  and  $S_2$  had less  $\sigma^2$  in response to glyphosate than the  $P_0$ -E, which suggested that the recurrent selection process reduced the inherent variability to glyphosate observed in the initial parental population. However, less difference in  $\sigma^2$  was observed when comparing the  $S_1$  and  $S_2$ . For instance, the  $\sigma^2$  of the hypocotyl length response to glyphosate was 0.27 for  $S_1$ -R and 1.84 for  $S_1$ -S, which was similar to that of 0.27 for  $S_2$ -R and 1.91  $S_2$ -S. Biomass response to glyphosate had a  $\sigma^2$  of 0.01, 0.02, 0.50, and 0.24 for  $S_1$ -R,  $S_1$ -S,  $S_2$ -R, and  $S_2$ -S, respectively.

While the variability to glyphosate was reduced in the selected *A. tuberculatus* populations, significant variability was still evident in the  $S_2$ -R and  $S_2$ -S. This observed persistent variability to glyphosate could be attributed to the reproductive nature *A. tuberculatus*. Genetic diversity is an inherent characteristic among intra- and inter-species of Amaranthaceae.<sup>51,52</sup> Recently, *Amaranthus rudis* J.D. Sauer and *A. tuberculatus* were grouped in a single-polymorphic species, where species with more genetic diversity occur in Ohio.<sup>47</sup> Because *A. tuberculatus* is an obligate-allogamous species, genetic recombination during mitosis could explain the phenotypic variability observed in the progeny of selected materials. Alternatively, resistance in *A. tuberculatus* could be determined by a polygenic event. Under this concept, alleles associated with resistance could recombine during mitosis,

produce different allele combinations, and generate a progeny with different levels of resistance, as observed in the segregant S<sub>2</sub>.

Finally, variation to glyphosate could be explained by the differential *EPSPS* expression at various *A. tuberculatus* phenological stages. Evidence for tissue-specific *EPSPS* expression was documented in petunia (*Petunia x hybrida*) where higher mRNA levels occurred in seedlings and flowers.<sup>53,54</sup> In addition, most plant genomes possess multiple *EPSPS* homologs. At least four *EPSPS* homologs were found on chromosome I and II of the *Arabidopsis thaliana* (L.) Heynh. genome through BLAST searches.<sup>55</sup> Furthermore, maize and sorghum (*Sorghum bicolor* (L.) Moench) possessed two *EPSPS* isoforms with similar structural, kinetic, and biochemical properties.<sup>56,57</sup> Apparently one isoenzyme was constitutively expressed, while the other appeared to be developmentally regulated. This suggested that the expression of *EPSPS* isoenzymes could represent an additional level of regulation of the shikimic acid pathway.<sup>57,58</sup>

#### **Phenotypes were characterized based on a three-level selection strategy**

Attempts to isolate stable resistant and susceptible *A. tuberculatus* lines through recurrent selection were unsuccessful given that variability for glyphosate efficacy was evident in the S<sub>2</sub>. The seedling assay was effective at screening large seed numbers; however, the method may be imperfect at elucidating glyphosate resistant and susceptible *A. tuberculatus* phenotypes. First, gene(s) that govern glyphosate resistance at the seedling stage may not be expressed at later *A. tuberculatus* phenological stage. Thus, resistant seedlings may develop into plants with similar response to glyphosate compared with plants that originated from seedlings identified as susceptible to glyphosate. Secondly, the seedling assay may not be completely efficient at identifying phenotypes at the seedling stage. Since the seedling assay was conducted for two weeks, readily emerging seedlings were probably exposed to higher glyphosate doses than those germinating during the second week of evaluation. This could lead to a misidentification of phenotypes and may explain the variability observed in the selected lines. Therefore, a three-level selection procedure was implemented to test whether the observed seedling resistance to glyphosate remained in mature *A. tuberculatus* plants.

S<sub>2</sub>-S and S<sub>2</sub>-R seeds were screened again at 10  $\mu$ M and 8 mM glyphosate, respectively, resulting in the isolation of 27 susceptible and 23 resistant *A. tuberculatus* plants (Table 1). Interestingly, disparity in the proportion of male and female plants was confirmed by the statistically significant *z* value for the S<sub>2</sub>-R population selected for resistance. Previously we attributed the disproportion of sex ratios in the S<sub>1</sub>-R to the small sample size (*n* = 12). However, the skewed male sex ratios in the S<sub>2</sub>-R selected for resistance suggested that glyphosate resistance in *A. tuberculatus* is probably associated with maleness. If sex in *A. tuberculatus* is chromosome-mediated, as suggested by Murray<sup>46</sup>, genes encoding male-specific protein(s) putatively involved in glyphosate resistance may be functional in the X chromosome but degenerate in the Y chromosome. Degenerate homologues in the non-pairing regions of Y chromosomes occur through nucleotide deletion or accumulation of repetitive sequences as reported in the dioecious white campion (*Silene latifolia* Poir.).<sup>59</sup>

#### **Stable lines isolated through asexually propagation (cloning)**

The 27 plants identified susceptible to glyphosate at the seedling stage were treated with 0.21 kg ha<sup>-1</sup> glyphosate when 10 cm tall, while the 23 resistant plants received a 1.21 kg ae ha<sup>-1</sup> rate at the same plant stage. Plants that developed  $\geq 30\%$  visual injury two weeks after application of 0.21 kg ha<sup>-1</sup> glyphosate were considered susceptible, whereas those treated with 1.21 kg ae ha<sup>-1</sup> and that developed  $\leq 30\%$  injury were classified resistant to glyphosate. This second level of selection resulted in the isolation of 6 ♀ and 6 ♂ susceptible and 3 ♀ and 15 ♂ glyphosate resistant plants. These plants were asexually propagated and the cloned materials tested for efficacy to glyphosate using the non-destructive assay. Additionally, 3 ♀ and 3 ♂ clones were prepared from the Paint Creek, OH *A. tuberculatus* population (P<sub>0</sub>-W). Consistent with expected results, detached P<sub>0</sub>-W stem-cuttings accumulated the highest levels of shikimic acid with 70.47 ( $\sigma_M = 1.34$ )  $\mu$ mol g<sup>-1</sup> of dry weight followed by the population selected for susceptibility to glyphosate with 42.42 ( $\sigma_M = 0.77$ )  $\mu$ mol g<sup>-1</sup> of dry weight. The population selected for resistance to glyphosate had the lowest levels of endogenous shikimic acid with 25.62 ( $\sigma_M = 0.48$ )  $\mu$ mol g<sup>-1</sup> of dry weight. These data supported the seedling and whole-plant assay results and confirmed that the response to glyphosate observed at the seedling stage was preserved in whole-plants.

Stem-cuttings were isolated from *A. tuberculatus* clones and the individual response to glyphosate determined non-destructively at the 0.86 kg ha<sup>-1</sup> rate. One male and female clone per population was identified and then propagated to further investigate the mechanism(s) of glyphosate resistance in *A. tuberculatus*. In the population selected for resistance, female clone #12 and male clone # 18 had the lowest endogenous shikimic acid levels and therefore were considered the most resistant within the population (Figure 4). Female clone # 7 and male clone # 10 in the population selected for susceptibility and female clone # 1 and male clone # 1 in P<sub>0</sub>-W were identified the most susceptible to glyphosate. Asexual propagation of *A. tuberculatus* facilitated the identification of resistant and susceptible individuals within selected populations and helped reduced the variability to glyphosate observed with sexual reproduction. Additionally, the method enabled propagation of *A. tuberculatus* materials with a confirmed phenotype to glyphosate suitable for studied to elucidate potential resistance mechanism(s).

#### **Several generations of selection may be required for resistance to evolve in the field**

Collectively the data herein presented confirmed that variability in response to glyphosate existed naturally in *A. tuberculatus* populations, and that the potential for the evolution of glyphosate resistance is significant. The frequency of resistant individuals within the Everly, IA *A. tuberculatus* populations was extremely low ( $< 4.0 \times 10^{-4}$ ) and thus required of high selection pressure to isolate resistant individuals. However, selection pressure under field conditions may be less stringent since survival of *A. tuberculatus* plants depends on several biotic and abiotic factors. Therefore, several generations of selection may be necessary to select *A. tuberculatus* genotypes with a stable trait for glyphosate resistance. Only moderate increases in the level of glyphosate resistance were reported after three cycles of selection at the whole-plant level in Illinois and Missouri *A. tuberculatus* populations.<sup>44</sup> Resistance in *L. multiflorum*, *L. rigidum*, *E. indica*, and *C. canadensis* evolved after several years of consistent glyphosate applications in the field.<sup>21,25,26,28</sup> The common motif in the confirmed cases of glyphosate resistance to date was the persistent and prolonged selection pressure imposed by glyphosate. This suggested that (1) the frequency of resistant individuals within the population was extremely low or (2) the trait for resistance

imposed a physiological penalty on plants. Evidence for the latter hypothesis was reported in petunia (*Petunia* sp.), where a single point mutation of Gly<sup>101</sup> to Ala encoded for an EPSPS with increased apparent dissociation constant ( $K_{i(app)}$ ) for glyphosate and apparent Michaelis-Menten constant ( $K_{m(app)}$ ) for PEP.<sup>60</sup> Gly<sup>101</sup> hydrogen bonds with the phosphate group of PEP; the added methyl group in the mutated EPSPS increased the steric hindrance on the phosphonate group of glyphosate compared to the more distant phosphate group in PEP.<sup>61</sup> This reduced affinity for PEP translated to a kinetically less efficient mutant EPSPS, thus plants containing this mutation may display an ecological fitness penalty compared to the wild type. While glyphosate resistance in *E. indica* was ascribed to a prolyl<sup>101</sup> to seryl point mutation in EPSPS, no clear mechanisms has been elucidated in the other resistant species.<sup>27</sup> Resistance to glyphosate may be conferred by several additive mechanism as reported in tolerant plants.<sup>18,19</sup>

At present, no confirmed case of glyphosate resistance has been reported in Midwest U.S. cropping systems. However, increases in the area planted with glyphosate resistant soybean and corn, and plans to release additional glyphosate resistant crops may increase the selection pressure and lead to the evolution of glyphosate resistant weeds.<sup>2</sup> Because glyphosate has no soil residual properties, multiple applications are often required to meet grower weed control expectations. Multiple applications summed to the current pervasive use in crops, will likely provide sufficient selection pressure for the evolution of glyphosate resistant weed populations. More research is needed to understand the impact of continuous glyphosate use on the dynamics of weed populations and the evolution of resistance. This information may relevant in developing effective weed control strategies and maintaining the productivity of current cropping systems in the U.S.

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**Table 1.** Summary of the divergent recurrent selection program to isolate glyphosate resistant (R) and susceptible (S) plants within the Everly, IA *Amaranthus tuberculatus* population (P<sub>0</sub>-E). Identification of R and S plants was performed at the seedling stage.

Material <sup>a</sup>	Selected lines	Estimated seeds selected <sup>b</sup>	Screening dose	Rescued seedlings	Relative frequency <sup>c</sup>	♀:♂ ratio at maturity	Relative frequency <sup>c</sup>	$z (P > z)^d$
P <sub>0</sub> -E	24	$3.54 \times 10^5$	32 $\mu$ M	376	$1.06 \times 10^{-3}$	24:29	$1.50 \times 10^{-4}$	0.69 (0.49)
P <sub>0</sub> -E	29	$3.84 \times 10^5$	3.2 mM	159	$4.14 \times 10^{-4}$	7:6	$3.38 \times 10^{-5}$	0.28 (0.78)
S <sub>1</sub> -S	24	$2.51 \times 10^5$	10 $\mu$ M	320	$1.27 \times 10^{-3}$	16:21	$1.47 \times 10^{-4}$	0.82 (0.41)
S <sub>1</sub> -R	7	$1.38 \times 10^5$	10 mM	113	$8.19 \times 10^{-4}$	0:12	$8.70 \times 10^{-5}$	3.46 (< 0.01)
BC <sub>R</sub>	7	$2.01 \times 10^5$	8 mM	121	$6.02 \times 10^{-4}$	7:11	$8.96 \times 10^{-5}$	0.94 (0.35)
S <sub>2</sub> -S	16	$1.73 \times 10^5$	10 $\mu$ M	242	$1.40 \times 10^{-3}$	12:15	$1.56 \times 10^{-4}$	0.58 (0.56)
S <sub>2</sub> -R	7	$1.37 \times 10^5$	8 mM	209	$1.53 \times 10^{-3}$	5:18	$1.68 \times 10^{-4}$	2.71 (< 0.01)

<sup>a</sup> = P<sub>0</sub>-E, parental Everly IA population; S<sub>1</sub>-S, susceptible first recurrent generation; S<sub>1</sub>-R, resistant first recurrent generation; BC<sub>R</sub>, back cross of S<sub>2</sub>-R to S<sub>1</sub>-R; S<sub>2</sub>-S, susceptible second recurrent generation; S<sub>2</sub>-R, resistant second recurrent generation.

<sup>b</sup> = based on a mean *A. tuberculatus* seed weight of 20.8 mg per 100 seeds

<sup>c</sup> = proportion of confirmed R or S individuals relative to the estimated seed selected

<sup>d</sup> = the one-tailed sign test assessed whether the observed sex ratios in selected lines were equally proportioned 1:1 (♀:♂)

**Table 2.** Rate response summary of the pristine Paint Creek, OH (P<sub>0</sub>-W), parental Everly, IA (P<sub>0</sub>-E) populations, and first (S<sub>1</sub>) and second (S<sub>2</sub>) recurrent *Amaranthus tuberculatus* generations selected for susceptibility (S) and resistance (R) to glyphosate.

Parameter	Material <sup>a</sup>	Model	GR <sub>50</sub> <sup>b</sup>	CI <sup>c</sup>	LOF <sup>d</sup>	R <sup>2</sup> <sub>(pseudo)</sub> <sup>d</sup>	λ <sub>50</sub>   <sup>e</sup>	P value <sup>e</sup>
hypocotyl	P <sub>0</sub> -E	Brain-Cousens	1.563	1.122–2.004	0.78	0.78	0.004	0.319
	P <sub>0</sub> -W	Log-logistic	1.567	1.142–1.993				
	S <sub>1</sub> -S	Log-logistic	0.788	0.570–1.006	0.34	0.84	3.918	0.334
	BC <sub>R</sub>	Log-logistic	4.706	3.187–6.225				
	S <sub>2</sub> -S	Log-logistic	0.592	0.347–0.837	0.21	0.84	8.061	0.018
	S <sub>2</sub> -R	Log-logistic	8.652	6.623–10.682				
radicle	P <sub>0</sub> -E	Log-logistic	0.074	0.050–0.097	0.16	0.81	0.193	0.165
	P <sub>0</sub> -W	Log-logistic	0.267	0.195–0.338				
	S <sub>1</sub> -S	Log-logistic	0.395	0.247–0.543	0.07	0.89	1.919	0.067
	BC <sub>R</sub>	Brain-Cousens	2.314	1.805–2.824				
	S <sub>2</sub> -S	Log-logistic	0.113	0.080–0.146	0.52	0.88	1.664	0.283

	S <sub>2</sub> -R	Log-logistic	1.777	1.186–2.368				
biomass	P <sub>0</sub> -E	Log-logistic	0.620	0.521–0.719	0.44	0.69	0.381	0.254
	P <sub>0</sub> -W	Log-logistic	0.239	0.153–0.324				
	S <sub>1</sub> -S	Log-logistic	0.532	0.476–0.588	< 0.01	0.89	0.354	0.004
	BC <sub>R</sub>	Log-logistic	0.886	0.831–0.941				
	S <sub>2</sub> -S	Log-logistic	0.289	0.145–0.432	0.75	0.44	0.624	< 0.001
height	S <sub>2</sub> -R	Log-logistic	0.913	0.713–1.113				
	P <sub>0</sub> -E	Log-logistic	0.473	0.391–0.554	0.75	0.73	0.104	0.371
	P <sub>0</sub> -W	Log-logistic	0.369	0.294–0.444				
	S <sub>1</sub> -S	Log-logistic	0.389	0.330–0.448	0.41	0.86	0.483	0.133
	BC <sub>R</sub>	Log-logistic	0.872	0.805–0.940				
	S <sub>2</sub> -S	Log-logistic	0.272	0.221–0.322	0.82	0.76	0.321	0.543
	S <sub>2</sub> -R	Log-logistic	0.593	0.496–0.690				

<sup>a</sup> = P<sub>0</sub>-E, parental Everly IA population; S<sub>1</sub>-S, susceptible first recurrent generation; S<sub>1</sub>-R, resistant first recurrent generation;

BC<sub>R</sub>, back cross of S<sub>2</sub>-R to S<sub>1</sub>-R; S<sub>2</sub>-S, susceptible second recurrent generation; S<sub>2</sub>-R, resistant second recurrent generation.

<sup>b</sup> = glyphosate dose in mM (hypocotyl; radicle) or rate in kg ae ha<sup>-1</sup> (biomass; height) that reduced by 50% the evaluated plant parameter

<sup>c</sup> = 95% lower and upper confidence interval of the  $GR_{50}$  value estimated by NLIN

<sup>d</sup> = Lack-of-fit (LOF) tests and pseudo coefficients of determination [ $R^2_{(pseudo)}$ ] estimated the proportion of the total variation explained by the model

<sup>e</sup> =  $|\lambda_{50}|$  quantifies the difference between the estimated  $GR_{50}$  parameters by parameterization of the Brain-Cousens model; the  $P$  value indicates the statistical significance of this difference



**Table 3.** Estimate glyphosate lethality in the pristine Paint Creek, OH (P<sub>0</sub>-W), parental Everly, IA (P<sub>0</sub>-E) populations, and first (S<sub>1</sub>) and second (S<sub>2</sub>) recurrent *Amaranthus tuberculatus* generations selected for susceptibility (S) and resistance (R) to glyphosate.

Material <sup>a</sup>	LD <sub>50</sub> <sup>b</sup>	FL <sup>c</sup>	R/S <sup>d</sup>
P <sub>0</sub> -E	0.569	0.434–0.686	2.7
P <sub>0</sub> -W	0.213	0.107–0.294	
S <sub>1</sub> -S	0.547	0.379–0.684	1.4
BC <sub>R</sub>	0.779	0.610–0.947	
S <sub>2</sub> -S	0.320	0.217–0.435	2.6
S <sub>2</sub> -R	0.831	0.672–0.994	

<sup>a</sup> = P<sub>0</sub>-E, parental Everly IA population; S<sub>1</sub>-S, susceptible first recurrent generation; S<sub>1</sub>-R, resistant first recurrent generation; BC<sub>R</sub>, back cross of S<sub>2</sub>-R to S<sub>1</sub>-R; S<sub>2</sub>-S, susceptible second recurrent generation; S<sub>2</sub>-R, resistant second recurrent generation.

<sup>b</sup> = glyphosate rate in kg ae ha<sup>-1</sup> that inflicted 50% mortality in the *A. tuberculatus* population.

<sup>c</sup> = 95% lower and upper fiducial limits (FL) of the calculated LD<sub>50</sub> estimated by PROBIT.

<sup>d</sup> = calculated by dividing the LD<sub>50</sub> of the resistant material by that of the susceptible material.

**Table 4.** Association between endogenous shikimic acid concentration and *Amaranthus tuberculatus* height, biomass, or visual injury to glyphosate. Numbers in parenthesis indicate the probability of the calculated Spearman's correlation coefficient ( $r^2$ ).

Material <sup>a</sup>	Plant height <sup>b</sup>	Biomass <sup>c</sup>	Visual injury <sup>d</sup>
P <sub>0</sub> -E	DNP <sup>e</sup>	DNP <sup>e</sup>	DNP <sup>e</sup>
P <sub>0</sub> -W	-0.65 (< 0.01)	-0.74 (< 0.01)	0.74 (< 0.01)
S <sub>1</sub> -S	-0.79 (< 0.01)	-0.83 (< 0.01)	0.85 (< 0.01)
BC <sub>R</sub>	-0.85 (< 0.01)	-0.79 (< 0.01)	0.91 (< 0.01)
S <sub>2</sub> -S	-0.60 (< 0.01)	-0.62 (< 0.01)	0.70 (< 0.01)
S <sub>2</sub> -R	-0.43 (< 0.01)	-0.80 (< 0.01)	0.61 (< 0.01)

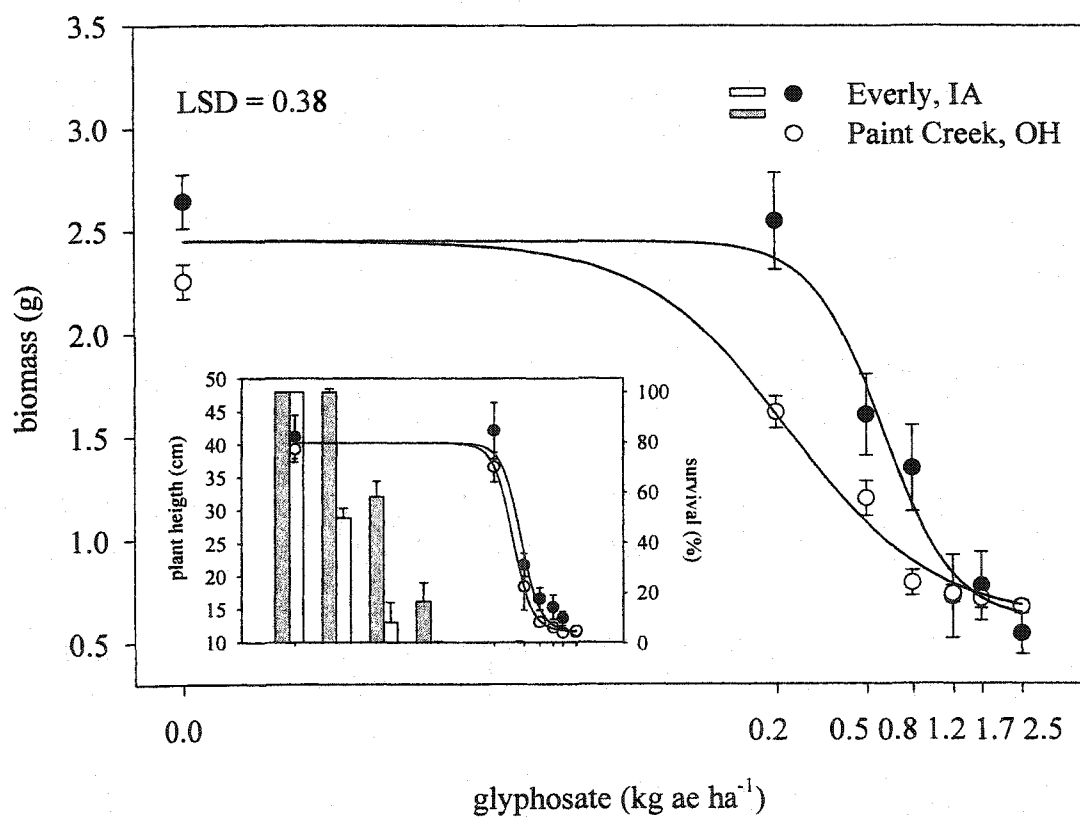
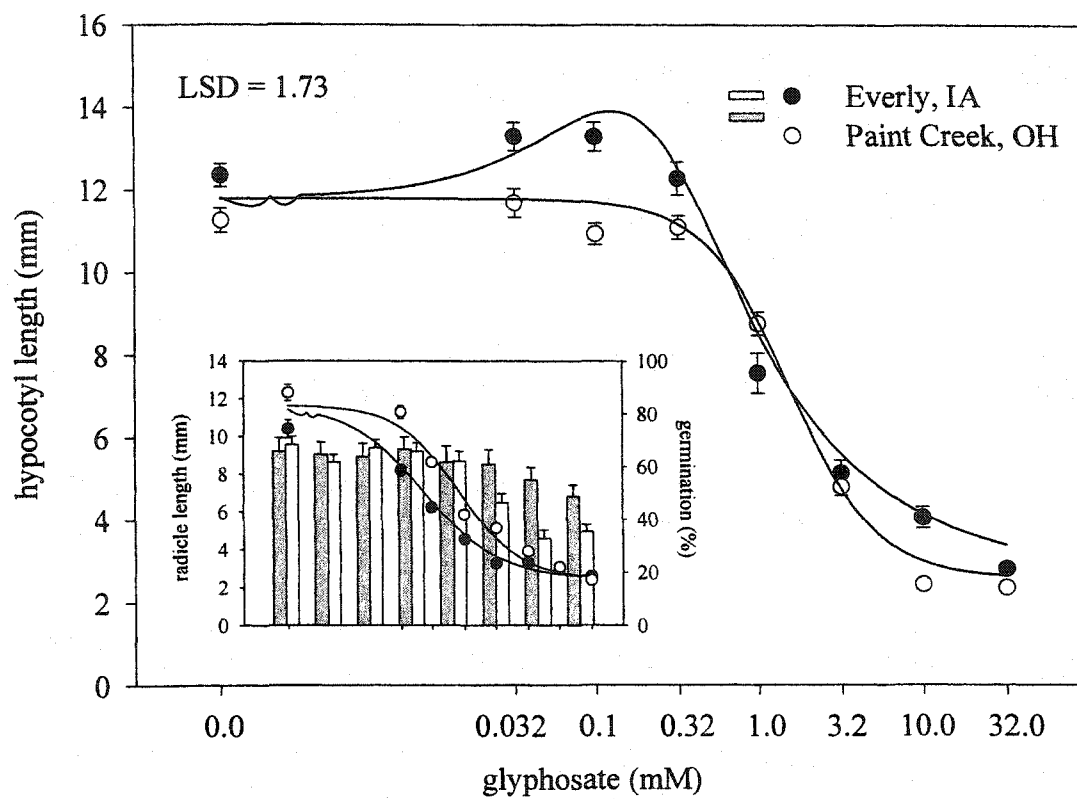
<sup>a</sup> = P<sub>0</sub>-E, parental Everly IA population; S<sub>1</sub>-S, susceptible first recurrent generation; S<sub>1</sub>-R, resistant first recurrent generation; BC<sub>R</sub>, back cross of S<sub>2</sub>-R to S<sub>1</sub>-R; S<sub>2</sub>-S, susceptible second recurrent generation; S<sub>2</sub>-R, resistant second recurrent generation.

<sup>b</sup> = distance in cm from the soil surface to the apex of *A. tuberculatus* plants.

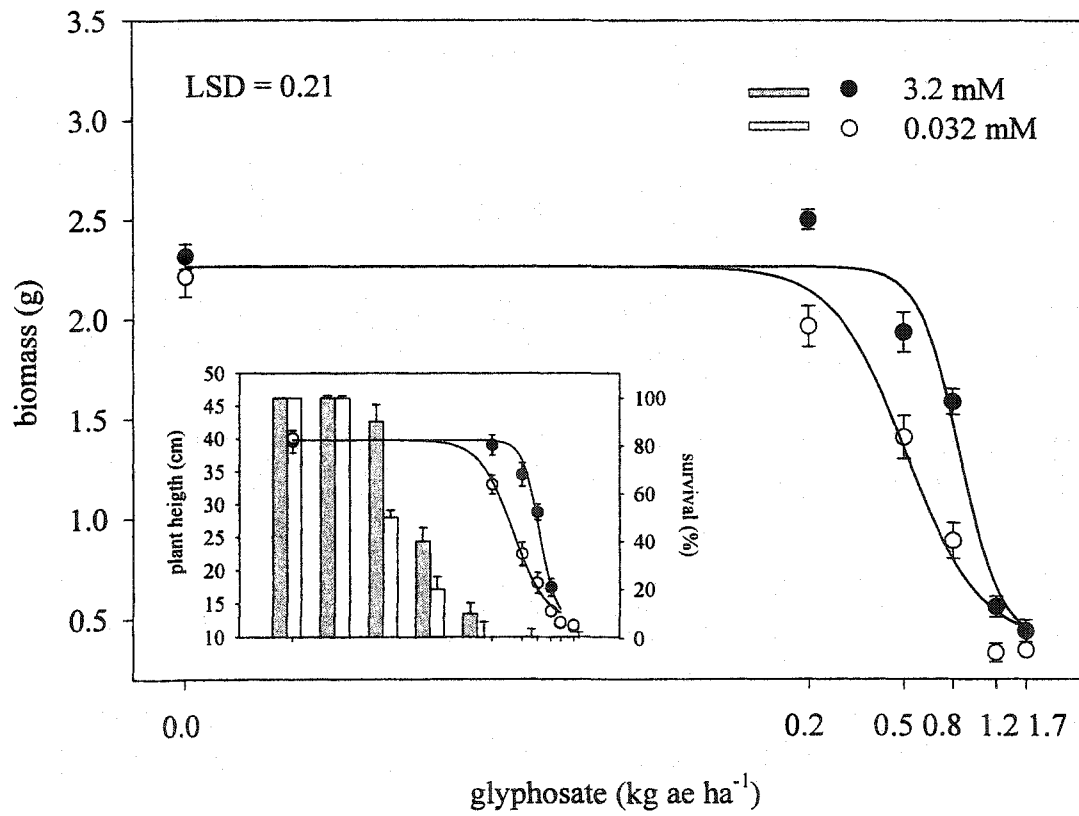
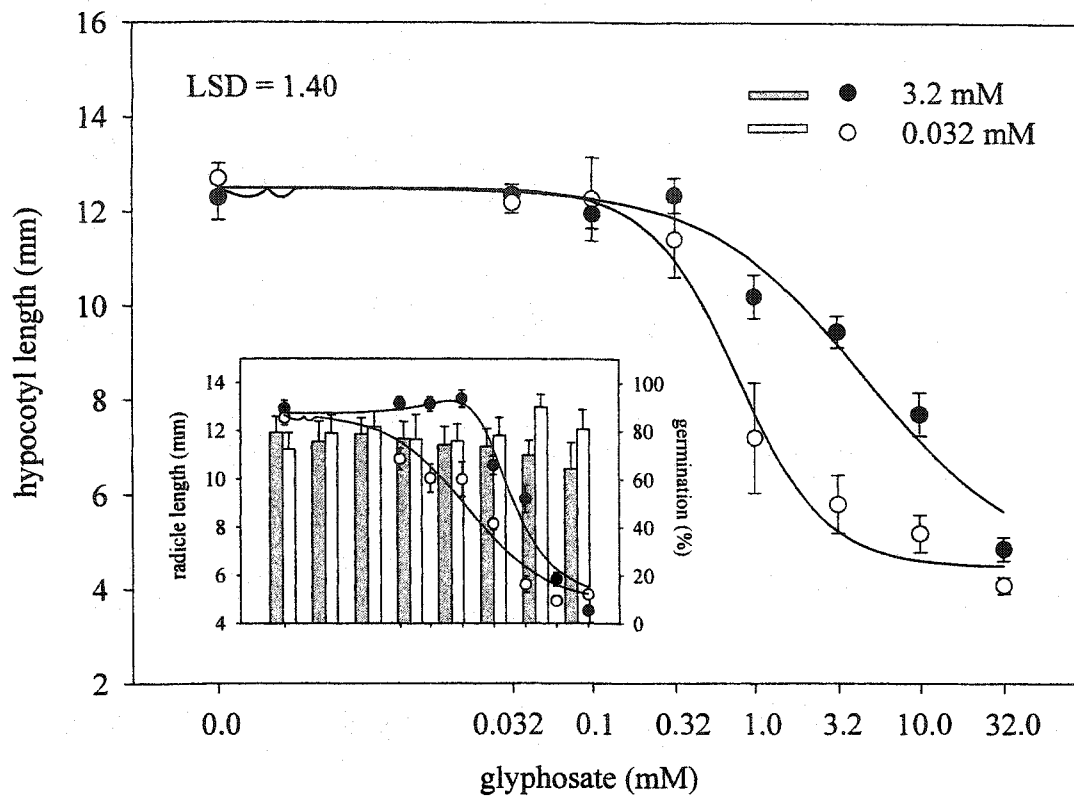
<sup>c</sup> = weight in g of dry *A. tuberculatus* plant tissue.

<sup>d</sup> = percent injury compared to control plants 14 days after glyphosate application.

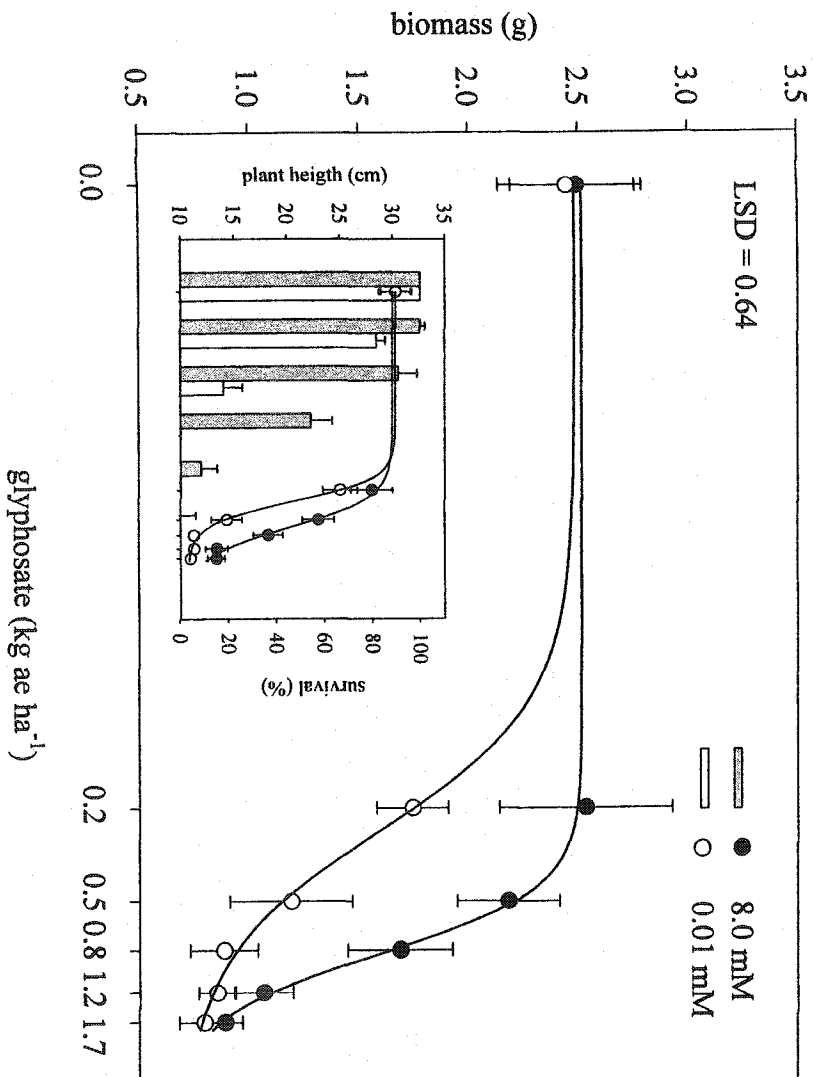
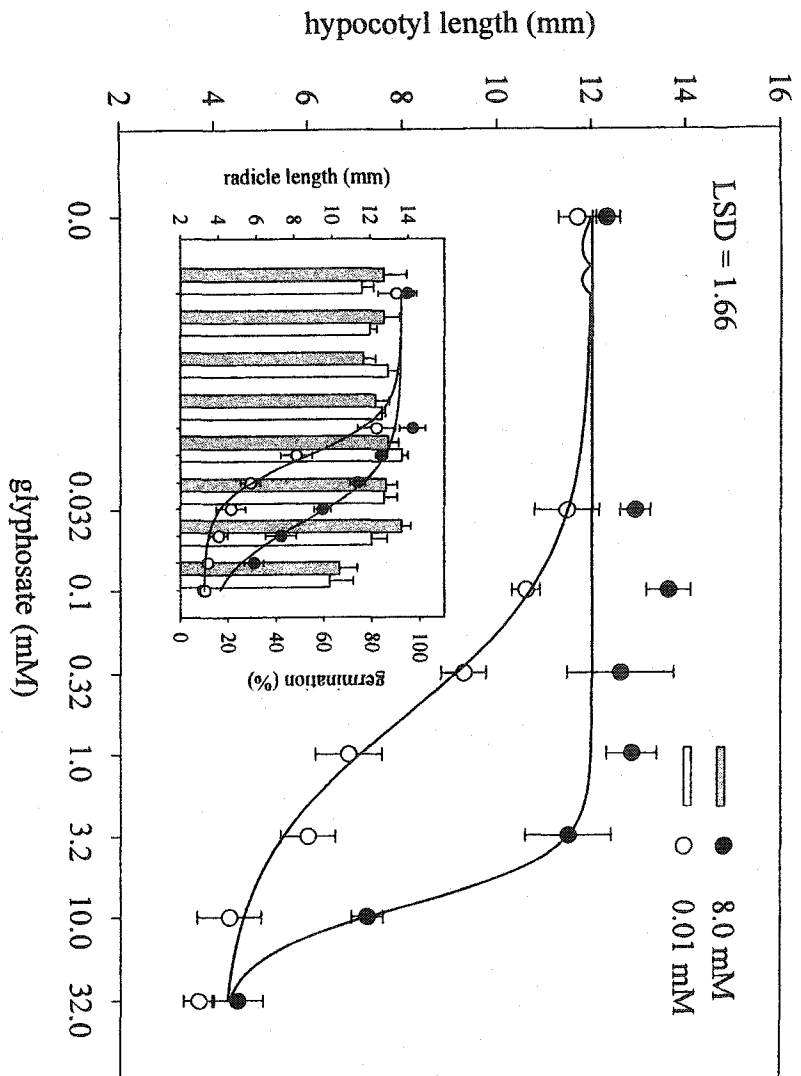
<sup>e</sup> = determination not performed.



**Figure 1.** Graph above: *Amaranthus tuberculatus* seedling dose responses of the parental Everly, IA (gray bars; ●) and pristine *A. tuberculatus* Paint Creek, OH (white bars; ○) populations to glyphosate. *Insert:* radicle length (circles) and germination (bars) as affected by glyphosate. Individual points or bars in the graph represent the mean of four separate experiments, each containing three replications ( $n = 12$ ). Graph below: whole-plant rate response of the parental *A. tuberculatus* Everly, IA (gray bars; ●) and pristine *A. tuberculatus* Paint Creek, OH (white bars; ○) populations to glyphosate. *Insert:* plant height (circles) and mortality (bars) associated with glyphosate rates. Data points represent the mean of two experiments with four replications ( $n = 8$ ). For both graphs, doses in inserts x axes correspond to those in the main graphs. Least significant difference at the  $P \leq 0.05$  level ( $LSD_{\alpha=0.05}$ ) was estimated by Fisher's test. Extensions on circles or bars designate the standard error associated with individual means ( $\sigma_M$ ).

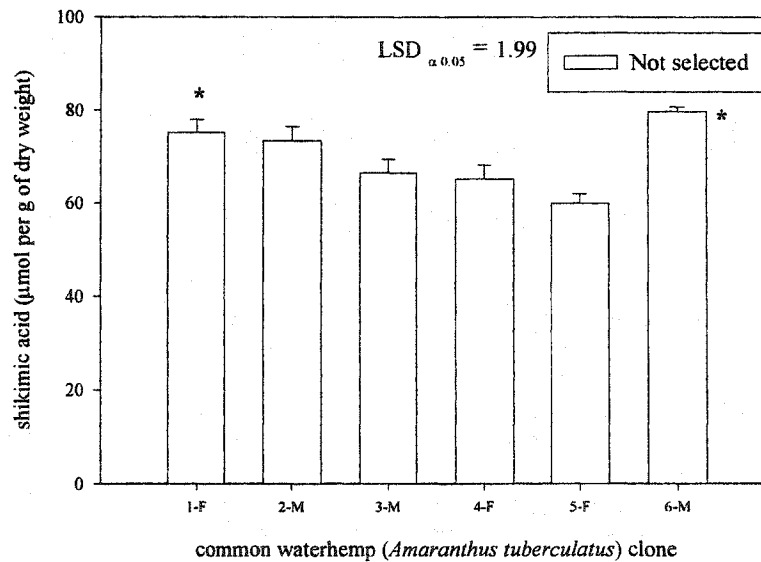
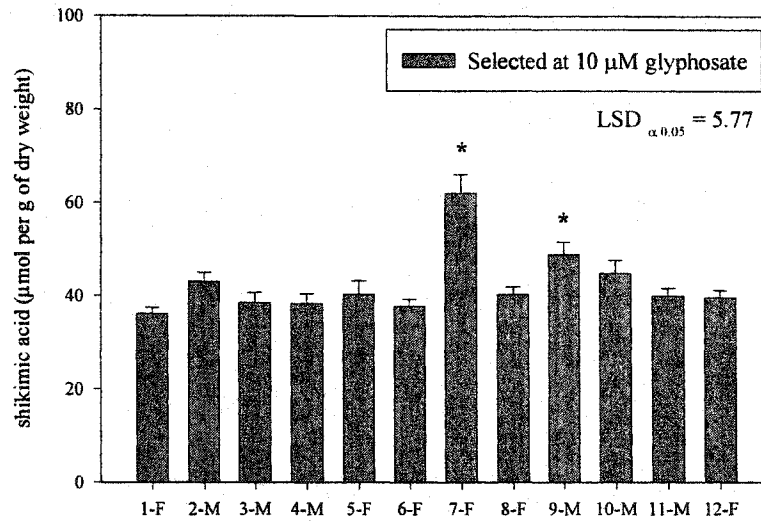
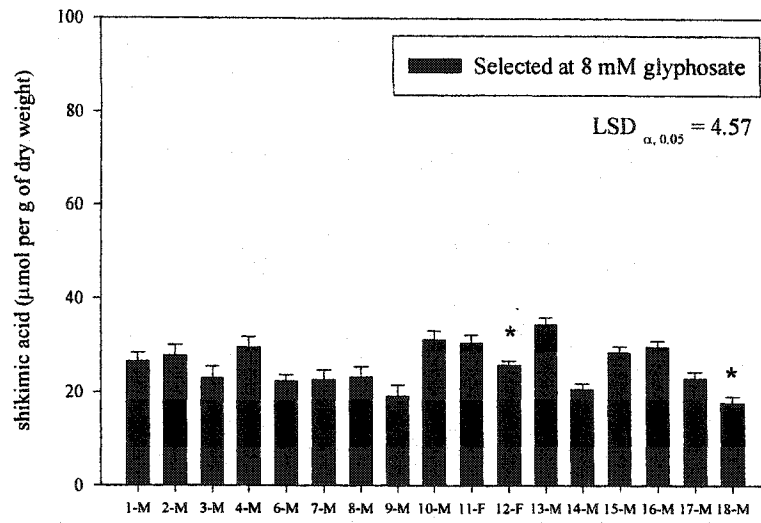


**Figure 2.** Graph above: *Amaranthus tuberculatus* seedling dose responses of the first recurrent material selected at 3.2 mM glyphosate (gray bars; ●) and 32  $\mu$ M glyphosate (white bars; ○). *Insert:* radicle length (circles) and germination (bars) as affected by glyphosate. Individual points or bars in the graph represent the mean of four separate experiments, each containing three replications ( $n = 12$ ). Graph below: *A. tuberculatus* whole-plant rate response of the first recurrent material selected at 3.2 mM glyphosate (gray bars; ●) and 32  $\mu$ M glyphosate (white bars; ○). *Insert:* plant height (circles) and mortality (bars) associated with glyphosate rates. Data points represent the mean of two experiments with four replications ( $n = 8$ ). For both graphs, doses in inserts  $x$  axes correspond to those in the main graphs. Least significant difference at the  $P \leq 0.05$  level ( $LSD_{\alpha=0.05}$ ) was estimated by Fisher's test. Extensions on circles or bars designate the standard error associated with individual means ( $\sigma_M$ ).



**Figure 3.** Graph above: *Amaranthus tuberculatus* seedling dose responses of the first recurrent material selected at 8.0 mM glyphosate (gray bars; ●) and 10  $\mu$ M glyphosate (white bars; ○). Insert: radicle length (circles) and germination (bars) as affected by glyphosate. Individual points or bars in the graph represent the mean of four separate experiments, each containing three replications ( $n = 12$ ). Graph below: *A. tuberculatus* whole-plant rate response of the first recurrent material selected at 8.0 mM glyphosate (gray bars; ●) and 10  $\mu$ M glyphosate (white bars; ○). Insert: plant height (circles) and mortality (bars) associated with glyphosate rates. Data points represent the mean of two experiments with four replications ( $n = 8$ ). For both graphs, doses in inserts  $x$  axes correspond to those in the main graphs. Least significant difference at the  $P \leq 0.05$  level ( $LSD_{\alpha=0.05}$ ) was estimated by Fisher's test. Extensions on circles or bars designate the standard error associated with individual means ( $\sigma_M$ ).





**Figure 4.** Non-destructive assay to identify the most resistant and susceptible *Amaranthus tuberculatus* lines within populations selected for resistance (8 mM glyphosate; black bars), susceptibility (10  $\mu$ M glyphosate; gray bar), or not selected (white bars). Phenotypic identification was performed based on the levels of shikimic acid in branched from clones treated with 0.86 kg ha<sup>-1</sup> glyphosate. One male (M) and female (F) clone per population was selected as indicated by the asterisk (\*). Each bar represents the mean of eight replications and a replication in time ( $n = 16$ ); extensions on bars designate the standard error associated with individual means ( $\sigma_M$ ).

### CHAPTER 3. GENETIC ANALYSIS OF EVOLVED GLYPHOSATE RESISTANCE IN HORSEWEED (*CONYZA CANADENSIS* (L.) CRONQ.)

A paper submitted to *Theoretical and Applied Genetics*<sup>1</sup>

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#### Abstract

Glyphosate [*N*-(phosphonomethyl) glycine] resistance was previously reported in a horseweed [*Conyza* (= *Erigeron*) *canadensis* (L.) Cronq.] population from Houston, DE ( $P_0^R$ ). Recurrent selection was performed on  $P_0^R$  since the population was comprised of susceptible (5%) and resistant (95%) phenotypes. After two cycles of selection at 2.0 kg glyphosate ha<sup>-1</sup>, similar responses were observed between the parental  $P_0^R$  and the first (RS<sub>1</sub>) and second (RS<sub>2</sub>) recurrent generations. This suggested that the RS<sub>2</sub> population comprised a near-homozygous glyphosate resistant line. Whole-plant rate responses estimated a four fold resistance increase to glyphosate between RS<sub>2</sub> and either a pristine Ames, IA ( $P_0^P$ ) or a susceptible *C. canadensis* population from Georgetown, DE ( $P_0^S$ ). The genetics of glyphosate resistance in *C. canadensis* was investigated by performing reciprocal crosses between RS<sub>2</sub> and either the  $P_0^P$  or  $P_0^S$  populations. Evaluations of the first (F<sub>1</sub>) and second (F<sub>2</sub>) filial generations suggested that glyphosate resistance was governed by an incompletely-dominant single locus gene (*R*-allele) located in the nuclear genome. The proposed genetic model was confirmed by back-crosses of the F<sub>1</sub> to plants that arose from achenes of the original RS<sub>2</sub>,  $P_0^P$ , or  $P_0^S$  parents. The autogamous nature of *C. canadensis*, the simple inheritance model of glyphosate resistance, and since heterozygous genotypes (F<sub>1</sub>) survived

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glyphosate rates well above those recommended by the manufacturer, predicted a rapid increase in frequency of the *R*-allele under continuous glyphosate selection. The impact of genetics on *C. canadensis* resistance management is discussed.

### Introduction

Since the commercial introduction in 1974, glyphosate [*N*-(phosphonomethyl) glycine] has become the most important herbicide worldwide primarily for its favorable characteristics – low mammalian toxicity, rapid degradation in the environment and resultant minimal ground water contamination, and effective–systemic activity on a diverse flora (Baylis 2000). Glyphosate inhibits 3-phosphoshikimate 1-carboxyvinyltransferase (EPSPS; EC 2.5.1.19), precluding the synthesis of important compounds derived from the shikimic acid pathway, instigating ultrastructure atrophy, and arresting protein synthesis (Mollenhauer et al. 1987; Muñoz–Rueda et al. 1986; Steinrücken and Amrhein 1980). Glyphosate resistance has been engineered through transformation with the metabolizing gene glyphosate oxidoreductase (*GOX*) (Barry et al. 1992), expression of an insensitive *EPSPS* (Padgett et al. 1991), *EPSPS* amplification (Shah et al. 1986), and enhanced *EPSPS* transcription (Klee et al. 1987). However, few resistance cases have evolved *in planta* despite the prolonged glyphosate use worldwide. The short half–life ( $t_{1/2}$ ) in the environment, unique biochemical characteristics, and complex molecular modifications required to engineer glyphosate–resistant crops were purported reasons for the low frequency of glyphosate resistance in weeds (Bradshaw et al. 1997).

The first confirmed glyphosate resistant weed was rigid ryegrass (*Lolium rigidum* Gaudin), where a 7– to 11–fold resistance evolved after 15 years of continuous glyphosate application (Powles et al. 1998). Differences in glyphosate uptake, translocation, or metabolism were disregarded as potential resistance mechanisms in *L. rigidum*, suggesting that resistance may be conferred by *EPSPS* overexpression, an insensitive EPSPS, or improper targeting of glyphosate to the loci of action (Feng et al. 1999; Lorraine–Colwill et al. 1999). More recently the mechanism of resistance in *L. rigidum* was credited to differences in cellular translocation of glyphosate (Lorraine–Colwill et al. 2003). Since reports of the *L. rigidum* biotype, glyphosate resistance was confirmed in goosegrass

[*Eleusine indica* (L.) Gaertner] (Lee and Ngim 2000), Italian ryegrass (*Lolium multiflorum* Lam.) (Pérez and Kogan 2003), hairy fleabane [*Conyza bonariensis* (L.) Cranq.], and buckhorn plantain (*Plantago lanceolata* L.) (Heap 2004). The resistance mechanism(s) in *L. multiflorum*, *C. bonariensis* and *P. lanceolata* are unknown to date; however despite ignoring the genetics, glyphosate resistance in *E. indica* was ascribed to a polymorphic EPSPS (Baerson et al. 2002b). At least one more glyphosate resistance mechanism, in addition to target site modification, apparently exists in another *E. indica* population from Malaysia (Ng et al. 2004). Plausible glyphosate resistance mechanisms include sequestration (Foley 1987), cellular compartmentation (Hetherington et al. 1998), differential translocation (Tucker et al. 1994), enhanced metabolism (Komoßa et al. 1992), increased transcription or extended  $t_{1/2}$  of the peptide encoded by EPSPS (Holländer-Czytko et al. 1992).

Horseweed [*Conyza* (= *Erigeron*) *canadensis* (L.) Cronq.] (Asteraceae) is a winter or summer annual North American native weed of importance in no-tillage crop production systems (Buhler and Owen 1997). *C. canadensis* is considered one of the ten most important herbicide-resistant weeds, evolving resistance to triazine, amide, bipyridilium, imidazolinone, and sulfonylurea herbicides in more than ten countries worldwide (Heap 2004). Northeast United States (US) farmers rely on glyphosate in combination with other residual herbicides for full-season *C. canadensis* management in glyphosate-resistant crops (VanGessel et al. 2001). Increased selection pressure resulted in inconsistent *C. canadensis* control with two split applications of 1.6 kg acid equivalents (ae) ha<sup>-1</sup> of glyphosate in glyphosate-resistant soybean [*Glycine max* (L.) Merr.] fields near Houston, DE. Whole-plant rate responses confirmed that the Houston biotype had an 8- to 13-fold resistance increase compared to a susceptible Georgetown, DE biotype; rates of 0.84 and 8.8 kg ha<sup>-1</sup> glyphosate were required to achieve control of the susceptible and resistant *C. canadensis* biotypes, respectively (VanGessel 2001). Noteworthy is the confirmation of least ten additional independent glyphosate-resistant *C. canadensis* populations throughout the US (Heap 2004).

Despite the global importance of glyphosate, limited information exists regarding the identity, frequency, and cellular location of genes associated with glyphosate resistance in plants. Herein we report on the genetic analysis of glyphosate resistance in the *C. canadensis*

population from Houston, DE, propose a model for the inheritance of the resistance gene (*R*-allele), and assess the level of allogamy between *Conyza* populations.

## Materials and methods

### Source of plant materials

The pristine *C. canadensis* population ( $P_0^P$ ) was obtained from the weed science seed collection at Iowa State University (ISU). ISU records indicated that  $P_0^P$  evolved without the selection pressure of glyphosate, in a wild-undisturbed area in the vicinity of Gateway Park in Ames, IA. The glyphosate resistant *C. canadensis* population ( $P_0^R$ ) was collected in a soybean field near Houston, DE where plants survived 1.6 kg glyphosate ha<sup>-1</sup>; a rate that effectively controlled the population in years prior. Evolution of the resistant population occurred in a no-tillage production system where glyphosate applied preplant and in glyphosate-resistant soybeans was the sole control method in 1998, 1999 and 2000. The glyphosate susceptible *C. canadensis* population ( $P_0^S$ ) was collected at the University of Delaware's Research and Education Center (UD-REC), near Georgetown, DE, in a field untreated with glyphosate for at least five years (VanGessel 2001). The  $P_0^P$ ,  $P_0^S$ , and  $P_0^R$  populations possessed stems with coarsely spreading hirsute and lacked purple tips on bracts; therefore, the populations were classified as *C. canadensis* var. *canadensis* (Gleason and Cronquist 1991).

### Achene storage and plant growth conditions

$P_0^P$  achenes were collected in 1994 by removing the inflorescence of mature *C. canadensis* plants in the field. The capitula were then allowed to dry at room temperature and achenes stored at 5 C until utilized in 2002. Similarly,  $P_0^R$  and  $P_0^S$  achenes was harvested from mature plants grown in the greenhouse, allowed to dry at room temperature, and stored at 5 C. For all three populations, achenes were planted in flats containing a peat:perlite:loam (1:2:1) soil-mix media and one week after seedling emergence, individual plants were transplanted to 12 cm diameter pots. Plants were grown in a greenhouse set at 28

to 35 C and 50 to 80% relative humidity (RH) diurnal and 20 to 25 C and 50% RH nocturnal conditions, and natural light was supplemented to a 16 hrs photoperiod with artificial illumination at  $600\text{--}1000\ \mu\text{mol}^{-2}\ \text{m}\ \text{s}^{-1}$  photosynthetic photon flux density (PPFD). Plants were irrigated as needed and fertilized (Miracle Gro Excel, Scott–Sierra Horticultural Products Co., 14111 Scottlawn Road, Marysville, OH 43041) one month after transplant. Prior to anthesis, plants used in crosses were transferred to a growth cabinet set at a 16 hr photoperiod, 25 C diurnal, 35 C nocturnal, 70% to 90% RH, and  $600\ \mu\text{mol}^{-2}\ \text{m}\ \text{s}^{-1}$  PPFD conditions.

### **Parental *Conyza* populations response to glyphosate**

#### *Classification of glyphosate resistant, intermediate-resistant, and susceptible phenotypes*

The manufacturer recommended glyphosate rate is  $0.85\ \text{kg ae ha}^{-1}$  of the isopropylamine salt of glyphosate (Roundup UltraMAX™, Monsanto, 700 Chesterfield Parkway North, St. Louis, MO 63198) sprayed on 10 cm diameter *C. canadensis* rosettes (Anonymous 2004). Typical glyphosate toxicity symptoms on *C. canadensis* included meristematic and leaf margin necrosis, leaf chlorosis especially in the area within veins, and arrested plant growth. Treatment of  $P_0^P$  or  $P_0^S$  rosettes at the 10 cm diameter stage with  $2.0\ \text{kg glyphosate ha}^{-1}$  caused visual herbicide injury levels  $\geq 70\%$  and uniform mortality 20 days after treatment (DAT). These parameters were adopted to define the glyphosate susceptible (S) phenotype. Conversely, treatment of  $P_0^R$  rosettes at the same stage and glyphosate rate resulted in marginal visual herbicide injuries ( $\leq 30\%$ ), thus prompting classification of the glyphosate resistant (R) phenotype. A third phenotype was identified in the progenies of crosses (explained below); the intermediate-resistant (IR) classification comprised plants that developed 31–69% visual injuries when treated as described for the other two classifications. Both R and IR phenotypes reached reproductive stage; however, IR phenotypes demonstrated slower growth rates than R phenotypes. No visual difference in growth rates was observed between R phenotypes and untreated *C. canadensis* plants. The reported phenotypic proportions within populations were estimated by ascribing *C. canadensis* plants treated with  $2.0\ \text{kg glyphosate ha}^{-1}$  to the parameters herein defined.

### *Rosette rate responses*

The performance of *C. canadensis* populations to glyphosate was evaluated by testing the response of 10 cm diameter rosettes to deionized water ( $\text{dH}_2\text{O}$ ; control), 0.42, 0.85, 1.69, 3.38, 6.77, or 13.54 kg glyphosate  $\text{ha}^{-1}$ . Glyphosate treatments were applied 30 cm from the plant canopy with an even flat fan nozzle (80015-E, TeeJet Spraying Systems, PO Box 7900, Wheaton, IL 60189-7900) in a  $\text{CO}_2$ -powered spray chamber (SB5-66, DeVries Manufacturing, Route 1 Box 184, Hollandale, MN 56045) delivering 187 l  $\text{ha}^{-1}$  at 2.8 kg per  $\text{cm}^2$ . Each treatment had four replications and an additional repetition was conducted in time ( $n = 8$ ). Treatments were sprayed in the morning and plants returned to the previously indicated greenhouse conditions. Glyphosate efficacy was evaluated 20 DAT by calculating the percent visual injury of treated plants compared to the  $\text{dH}_2\text{O}$ -treated control. Thereafter, biomass measurements were determined by cutting rosettes at the soil surface, drying at 35 C for 48 h in paper bags, and estimating the weigh of individual plant samples. Glyphosate efficacy was also assessed by monitoring the accumulation of endogenous shikimic acid (3R, 4S, 5R trihydroxy-1-cyclohexene-1-carboxylic acid) in a dry sub-sample per treated *C. canadensis* plants (explained below).

### *Endogenous shikimic acid extraction and determination*

A 0.5 g of biomass sub-sample was assayed in duplicate to estimate endogenous shikimic acid levels using a spectrophotometric protocol modified from Cromartie and Polge (2002). The dry *C. canadensis* tissue was ground with 2.5 mm glass beads for 10 min in a BeadBeater<sup>®</sup> (BioSpec Products, P.O. Box 788, Bartlesville, Oklahoma 74005-0788) and shikimic acid extracted in a 1:10 tissue:0.25 N HCl phase for 48 h at 5 C. The samples were then centrifuged at 15,000 g for 15 min to precipitate cell debris and a 5–10  $\mu\text{L}$  aliquot sample oxidized with 22 mM periodate plus sodium *meta*-periodate for 45 min at 45 C. The shikimic acid chromophore was generated by adding 1 M NaOH and immediately stabilized with 56 mM  $\text{Na}_2\text{SO}_3$ . Finally, absorbance was detected at 382 nm ( $A_{382}$ ) and a previously prepared standard curve at 1 to 60  $\mu\text{mol ml}^{-1}$  shikimic acid (Sigma-Aldrich, 3050 Spruce Street, Saint Louis, MO 63103) was used to convert  $A_{382}$  data to  $\mu\text{mol}$  shikimic acid equivalents  $\text{g}^{-1}$  dry weight.



## Genetic analysis of glyphosate resistance

### *Recurrent selection of the resistant C. canadensis material*

Greenhouse evaluations indicated that the  $P_0^P$  and  $P_0^S$  populations were uniformly susceptible to glyphosate, while approximately 95% of the individuals in the  $P_0^R$  population were resistant to glyphosate. Therefore, a stable-homogenous resistant population was isolated through two cycles of recurrent selection.  $P_0^R$  rosettes were treated with 2.0 kg glyphosate  $ha^{-1}$  at the 10 cm diameter stage, evaluated for efficacy 20 DAT, and ten plants with a resistant phenotype allowed to grow and self-pollinate in the greenhouse. The resulting population comprised the first recurrent generation ( $RS_1$ ). Accordingly, the second recurrent generation ( $RS_2$ ) was isolated by undergoing another cycle of selection on  $RS_1$  material as indicated in this section. Intraspecific and back-crosses were conducted with  $P_0^P$ ,  $P_0^S$ , and  $RS_2$  plants with a confirmed phenotype (explained below).

### *Phenotypic confirmation of parents utilized in crosses*

Both  $P_0^P$  and  $P_0^S$  populations were not selected since rate responses confirmed that these populations were susceptible for glyphosate. Treatment of 10 cm diameter  $P_0^P$  or  $P_0^S$  rosettes with 0.4 kg glyphosate  $ha^{-1}$  resulted in  $\leq 60\%$  visual injuries and a reduction in rosette growth rate compared to untreated *C. canadensis* rosettes. However,  $P_0^P$  or  $P_0^S$  rosettes treated with the 0.4 kg  $ha^{-1}$  rate recovered from injuries within 2 to 4 weeks and reached reproductive stage. Concomitantly, the 0.4 kg  $ha^{-1}$  sub-lethal glyphosate rate permitted non-destructive confirmation of the susceptible parents utilized in the interspecific and back-crosses (explained below). The resistant parents were confirmed by treating 10 cm diameter  $RS_2$  rosettes with 2.0 kg glyphosate  $ha^{-1}$  and evaluating efficacy to the herbicide 20 DAT; rosettes with  $\leq 30\%$  injury were used in the crosses.

### *Estimates of allogamy through assisted intraspecific crosses*

Ten  $RS_2$  and  $P_0^S$  rosettes confirmed phenotypically, as indicated in the previous section, were grown in the greenhouse and transferred to the growth cabinet approximately

two weeks prior to anthesis. Assisted crosses were performed between the glyphosate resistant and susceptible phenotypes to assess the levels of cross-pollination (alogamy) between *C. canadensis* plants. Ten RS<sub>2</sub> and P<sub>0</sub><sup>S</sup> plant-pairs (families) were allowed to grow in isolation. At anthesis, the inflorescences of RS<sub>2</sub> and P<sub>0</sub><sup>S</sup> plant-pairs were permitted to interact physically inside a PQ218 DelNet<sup>®</sup> bag (DelStar Technologies, 601 Industrial Drive, Middletown, DE 19709), thus restricting pollen release within the bag and limiting contamination from external pollen sources. Percent alogamy was estimated by determining the frequency of intermediate-resistant (IR) phenotypes within full-sibling populations.

#### *Intraspecific artificial crosses and back-crosses*

Given that *C. canadensis* has white pistillate ray and yellow perfect disk florets and that some self-fertilization can occur prior to anthesis (Weaver 2001), capitula emasculation was performed in artificial crosses to ensure the origin of spermatozoon used to fertilize the ovum in pollen-receptor plants. Emasculation was comprised of manually excising disk florets from unopened capitula with forceps under a magnifying lens; an estimated 50 capitula per plant were emasculated. The remaining non-emasculated capitula were removed from plants to limit self-fertilization. Approximately five days post emasculation the remaining pistillate florets became receptive, upon which stigmas were fertilized by gently rubbing the intact capitula of pollen-donor plants. Emasculated capitula were fertilized daily for one week, achenes allowed to mature in the mother plant, and removed when the pappus became visible. Finally, the mature achenes were germinated in soil-mix media and the resulting seedlings grown in the aforementioned greenhouse conditions. If emasculation was completely effective at eliminating self-fertilization in *C. canadensis*, emasculated capitula that matured in the absence of pollen would produce non-viable achenes. Therefore, the efficiency of emasculation was tested by assessing the non-germination of 50 emasculated capitula in each of ten *C. canadensis* plants that developed inside a DelNet<sup>®</sup> bag.

Twenty RS<sub>2</sub> and ten P<sub>0</sub><sup>P</sup> and P<sub>0</sub><sup>S</sup> plants previously confirmed phenotypically were crossed in reciprocal (R x S; S x R), totaling ten families per parent pair combination. The progeny of these crosses, representing the first filial generation (F<sub>1</sub>), were treated with 2.0 kg

glyphosate  $\text{ha}^{-1}$  and individual  $F_1$  plants classified phenotypically. One  $F_1$  plant per family was allowed to self-pollinate in isolation and the efficacy to glyphosate in the second filial generation ( $F_2$ ) was assessed through whole-plant rate responses and phenotypically at the single  $2.0 \text{ kg ha}^{-1}$  rate. To test the genetic model, one  $F_1$  plant per family was back-crossed to plants that derived from achenes of the original parents; these populations were labeled  $\text{BC}_R$  ( $\text{RS}_2$ ),  $\text{BC}_P$  ( $\text{P}_0^P$ ), or  $\text{BC}_S$  ( $\text{P}_0^S$ ) depending on the parent used in the back-cross. Three plants per each of the 20 generated families were randomly selected ( $n = 60$ ) to assess the rate response of  $F_1$  and  $F_2$  rosettes to glyphosate.

### Statistical analysis

All statistical analyses were conducted with the Statistical Analysis System (SAS) (SAS 2000). Replications in time were tested for patterns of covariance matrices that satisfied the Huynh-Feldt condition (option PRINTE) (Huynh and Feldt 1970). When the sphericity test confirmed that the covariances were type H,  $F$ -statistics tested the univariate analyses for within time effects and related interactions. Whole-plant rate responses were evaluated by analysis of variance (ANOVA) as a randomized complete block (RCB) design with four replications and repeated once in time (PROC GLM). When ANOVA identified significant population effects, mean separation was conducted with Fisher's least significant difference test (LSD) at the  $\alpha$  of 0.05. Visual injury data were converted to a dichotomous distribution following the classification for R ( $< 69\%$ ) and S ( $\geq 70\%$ ) phenotypes. The transformed injury data were then analyzed with a modified Newton-Raphson algorithm (PROC PROBIT) to estimate the glyphosate rate that inflicted 50% mortality in the population ( $LD_{50}$ ) (Collett 2002). In addition, biomass and shikimic acid data were subjected to log-logistic analysis (Gauss-Newton method) and the glyphosate rate that reduced plant growth by 50% ( $GR_{50}$ ) or instigated accumulation of half of the maximum detectable shikimic acid concentration ( $I_{50}$ ) was calculated (PROC NLIN) (Seefeldt et al. 1995). The non-linear model fit to the data was assessed graphically by the distribution of residuals and statistically by lack-of-fit (LOF) tests and pseudo-coefficients of determination [ $R^2_{(\text{pseudo})}$ ] (Schabenberger et al. 1999; Seefeldt et al. 1995). Biomass data model to the reparameterized

Brain–Cousens equation (Marquardt–Levenberg method) allowed for estimation of the probability ( $P$ ) for the absolute difference between two calculated  $GR_{50}$  values ( $|\lambda_{50}|$ ) (Schabenberger et al. 1999). The relationship strength between the estimated whole–plant rate response parameters and endogenous shikimic acid levels was determined by Spearman’s linear correlation analysis.

The phenotypic  $F_1$ ,  $F_2$ ,  $BC_R$ ,  $BC_P$ , and  $BC_S$  data were analyzed according to Cochran–Mantel–Haenszel statistics. The proposed genetic model was tested by comparing the observed R, IR, and S segregation ratios in full–siblings (families) against the expected Mendelian proportions for the model with a chi–square ( $\chi^2$ ) goodness–of–fit (GOF) test. Homogeneity  $\chi^2$  analysis was performed to ascertain whether combination of the segregation data within families was suitable.

## Results

### ***C. canadensis* populations responded distinctively to glyphosate**

#### *Recurrent selection increased the frequency of resistant phenotypes*

Greenhouse experimentation established that all 59  $P_0^P$  and 73  $P_0^S$  rosettes evaluated were uniformly susceptible to glyphosate at the 2.0 kg ha<sup>−1</sup> rate; in contrast, only 78 of 82  $P_0^R$  rosettes (95%) treated at this same rate demonstrated a resistant phenotype. This suggested that the  $P_0^R$  population was comprised of susceptible phenotypes, in addition to homozygous and probably heterozygous resistant genotypes. Thus, recurrent selection was imposed on  $P_0^R$  plants to isolate a stable–homogenous resistant population. Evaluations of  $RS_1$  and  $RS_2$  plants confirmed that all 79 and 84 rosettes evaluated, respectively, had a resistant phenotype at 2.0 kg glyphosate ha<sup>−1</sup> rate. Recurrent selection results therefore suggested that the  $RS_2$  population comprised a homogenously resistant line. Resistant  $RS_2$  phenotypes demonstrated only limited injury to glyphosate at the 2.0 kg ha<sup>−1</sup> rate, had growth rates analogous to the untreated  $P_0^P$  or  $P_0^S$  rosettes, and were able to complete the reproductive cycle. To investigate the rate response of the parental and selected populations to glyphosate, the sums of squares and crossproducts (SSCP) matrix of experiments conducted in time were first estimated to assess the suitability for a combined data analysis.

These estimates provided a statistically significant partial correlation estimate for biomass ( $r^2 = 0.33$ ;  $P < 0.001$ ) and visual injury ( $r^2 = 0.54$ ;  $P < 0.001$ ), suggesting a strong relationship strength between the measurements acquired in time. Concurrently, multivariate ANOVA test for the null hypothesis ( $H_0$ ) of no time effect resulted in non-significant estimates for biomass (Wilks'  $\lambda = 0.99$ ;  $P = 0.66$ ) and visual injury (Wilks'  $\lambda = 0.99$ ;  $P = 0.62$ ); therefore, a negligible effect of replication in time was inferred and data were combined in henceforth analyses.

*Parental populations represent near-homozygous lineages*

Adequacy of the log-logistic model for describing the population response to increasing glyphosate rates was calculated by LOF and coefficient of determination estimates. Satisfactory overall quality model fit was confirmed by the resulting  $R^2_{(\text{pseudo})}$  values for biomass (0.73) and shikimic acid (0.72) measurements, and the LOF test (biomass,  $F = 0.58$ ,  $P = 0.97$ ; shikimic acid,  $F = 0.47$ ,  $P = 0.99$ ). Therefore, it was inferred that parameters estimated by the log-logistic model predicted dependably the performance of *C. canadensis* populations to glyphosate. Approximately 0.5 kg glyphosate ha<sup>-1</sup> was the effective rate reducing plant growth by 50% in either  $P_0^P$  or  $P_0^S$  populations (Table 1), and the absolute difference between the estimated  $GR_{50}$  values was not statistically different ( $|\lambda_{50}| = 0.03$ ;  $F_{\text{obs}} = 0.82$ ;  $P = 0.96$ ). The glyphosate rate required to inflict 50% mortality on either  $P_0^P$  or  $P_0^S$  populations was also similar (Table 1). Hence, the performance to glyphosate of both the pristine Ames, IA and susceptible Georgetown, DE populations was considered equivalent.

At least a four fold and seven fold resistance increase to glyphosate, respectively, was estimated in the  $P_0^R$  population compared to either  $P_0^P$  or  $P_0^S$  based on biomass ( $GR_{50}$ ) or mortality ( $LD_{50}$ ) responses (Table 1). More visual injury was also recorded in susceptible, compared to resistant phenotypes, above the 0.85 kg ha<sup>-1</sup> glyphosate rate (Figure 1). It was of interest to ascertain whether the estimated  $GR_{50}$  value for the resistant and susceptible populations differed statistically. Therefore,  $|\lambda_{50}|$  were calculated for contrasts between  $P_0^R$

and  $P_0^P$  or  $P_0^S$ , resulting in values of  $1.61 \text{ kg ha}^{-1}$  ( $F_{\text{obs}} = 1.33$ ;  $P = 0.006$ ) and  $1.58 \text{ kg ha}^{-1}$  ( $F_{\text{obs}} = 1.27$ ;  $P = 0.016$ ), respectively. The statistical significance of these contrasts confirmed that the *C. canadensis* Houston, DE population differed in response to glyphosate from the Georgetown, DE and Ames, IA populations. Other confirmed cases of glyphosate resistance ascribed  $GR_{50}$  values of  $1.2 \text{ kg ha}^{-1}$  in *L. multiflorum*,  $4.9 \text{ kg ha}^{-1}$  in *E. indica*, and  $4.6$  to  $5.1 \text{ kg ha}^{-1}$  in *L. rigidum* (Lee and Ngim 2000; Lorraine-Colwill et al. 2001; Pérez and Kogan 2003).

Since  $P_0^R$  was comprised of susceptible phenotypes, and if resistance to glyphosate in *C. canadensis* was inherited as a dominant trait, recurrent selection would increase the frequency of resistant individuals and therefore the overall population response to glyphosate. To contemplate this possibility, rate responses were conducted on the  $RS_1$  and  $RS_2$  populations and response parameters were compared to those of the original  $P_0^R$  population. Not only the estimated  $GR_{50}$  and  $LD_{50}$  values overlapped at the 95% confidence and fiducial intervals and suggested similar population responses to glyphosate (Table 1), but  $|\lambda_{50}|$  comparisons were non-significant. These results reaffirmed the notion of parallel performances of  $P_0^R$  and  $RS_1$  ( $F_{\text{obs}} = 0.82$ ;  $P = 0.96$ ) and  $P_0^R$  and  $RS_2$  ( $F_{\text{obs}} = 0.89$ ;  $P = 0.85$ ) to glyphosate. Hence, the  $RS_2$  populations was considered near-homozygous resistant given that the parental and selected populations performed similarly to glyphosate.

#### *Less shikimic acid accumulates in resistant plants*

In plants, glyphosate causes cytoplasmic accumulation of the substrate and unphosphorylated substrate of EPSPS at a 1:20 proportion of 3-phosphoshikimate (3PS):shikimic acid (Gout et al. 1992). Ultimately, putative phosphorylases hydrolyzed the phosphoryl group in 3PS and the aromatic compound is accumulated as shikimic acid in cell vacuoles (Holländer-Czytko and Amrhein 1983). Whole-plant response to glyphosate can therefore be confirmed by monitoring endogenous shikimic acid concentrations (Harring et al. 1998). In addition, shikimic acid levels may serve as an indirect indicator of the level of EPSPS inhibition by glyphosate.

In the absence of glyphosate, *C. canadensis* rosettes contained extractable shikimic acid concentrations of 18 to 25  $\mu\text{mol g}^{-1}$  of dry tissue across all populations. These basal levels increased sigmoidally with increasing glyphosate rates to an approximate maximum of 113 to 133  $\mu\text{mol shikimic acid g}^{-1}$  of dry tissue at 20 DAT (Figure 1). The glyphosate rate required to inhibit half of EPSPS in the  $P_0^P$  or  $P_0^S$  populations was close to 2.0  $\text{kg ha}^{-1}$ , in contrast to 3.1 to 4.4  $\text{kg ha}^{-1}$  required for the resistant or recurrent selected populations (Table 1). Marginal differences in shikimic acid levels were observed 20 DAT at the 0.42 or 0.85  $\text{kg glyphosate ha}^{-1}$  rates, while maximum differences occurred at 3.38  $\text{kg glyphosate ha}^{-1}$  (Figure 1). This confirmed that EPSPS in  $RS_2$  was less inhibited at glyphosate rates  $> 0.85 \text{ kg ha}^{-1}$  compared to  $P_0^P$  or  $P_0^S$ . Patterns of shikimic acid accumulation also correlated negatively with biomass and positively with visual injury assessments (Table 1). Mueller et al. (2003) reported that in a glyphosate resistant *C. canadensis* biotype from Tennessee, shikimic acid levels decreased significantly 4 DAT compared to 2 DAT at the 0.84  $\text{kg ha}^{-1}$  glyphosate rate. *C. canadensis* possesses three EPSPS isoforms (Montgomery et al. 2003), each with apparently different kinetic constants, thus potentially explaining the differential EPSPS inhibition reported in resistant plants. A remote alternative purports that resistant plants possess GOX-like proteins that metabolize glyphosate (Mueller et al. 2003).

### ***C. canadensis* is essentially autogamous**

Estimates of emasculation efficiency suggested that some ( $< 1\%$ ) self-fertilization (autogamy) may occur prior to capitula unveil (Table 2). An alternative explanation to these results was that some pollen was released while performing excision of perfect florets during emasculation. Approximately 45% of *C. canadensis* florets are self-fertilized under normal conditions (Zelaya et al., this issue). Thus the emasculation method was approximately 98% effective at preventing autogamy in *C. canadensis*. Estimates of assisted cross pollination (allogamy) across families ranged from 0% to 14% in the  $RS_2$  to  $P_0^P$  or  $P_0^S$  cross and 0% to 10% in the reciprocal  $P_0^P$  or  $P_0^S$  to  $RS_2$  cross (Table 2). Weaver (2001) reported an average 4% allogamy, ranging from 1.2% to 14.5%, in a paraquat resistant *C. canadensis* biotype. Assisted crosses estimated allogamy under ideal conditions; in nature, inflorescent proximity,

abiotic factors as wind, and biotic agents as insects may modulate allogamy dynamics between *C. canadensis* plants.

### **The *R*-allele is nuclear encoded**

Artificial crosses provided an estimate of the intraspecific compatibility within *C. canadensis* and ascertained whether glyphosate resistance was maternally inherited. Across all families and artificial reciprocal crosses, > 92% of treated rosettes demonstrated an IR phenotype (Table 2). This confirmed that *C. canadensis* plants were overall genetically compatible. The unexpected levels of susceptible and resistant phenotypes in the  $F_1$  were attributed to the inefficiency (2%) associated with emasculation or autogamy prior to anthesis. Artificial reciprocal crosses also established that the *R*-allele was pollen borne since the vast majority of  $F_1$  rosettes displayed an IR phenotype. In the event of cytoplasmic inheritance of glyphosate resistance, susceptible phenotypes would have predominated the  $RS_2$  to  $P_0^P$  or  $P_0^S$  artificial cross. With the exception of some instances in resistance to triazine herbicides, the predominant cases of herbicide resistance are conferred by nuclear gene(s) (Gasquez 1997).

### **Glyphosate resistance in *C. canadensis* follows the 1:2:1 model**

Segregation ratios were monitored in  $F_2$  full-siblings to ascertain the number of genes and based on phenotypic frequencies, a model was constructed to explain the inheritance of glyphosate resistance in *C. canadensis*. The purported genetic model was tested by back-crosses of  $F_1$  plants to a progenitor from the original  $RS_2$ ,  $P_0^P$  or  $P_0^S$  parent. Moreover, rate responses were conducted to confirm intermediacy of the putative heterozygous  $F_1$  to glyphosate. Efficacy trials of  $F_2$  full-siblings at 2.0 kg glyphosate  $ha^{-1}$  identified R, IR, and S phenotypes, as defined earlier, within each family. Visual assessments suggested that glyphosate resistance in *C. canadensis* segregated following partially-dominant Mendelian genetics consistent with a single gene effect.  $F_2$  families generated from the  $RS_2$  to  $P_0^P$  cross had observed phenotypic ratios that converged to the expected 1:2:1 proportion predicted by Mendelian genetics ( $\chi^2 < 2.79$ ,  $P > 0.25$ ) (Table 3). Concomitantly, GOF analysis for the



reciprocal  $P_0^P$  to  $RS_2$  families and the combined homogenous data set for all  $F_2$  families ( $\chi^2 = 0.44$ ,  $P = 0.80$ ) provided non-significant  $\chi^2$  values, reaffirming appropriateness of the incompletely-dominant monogenic model. Results from the  $\chi^2$  homogeneity test permitted combined analysis of the back-cross data; GOF results were consistent with the expected 1:1 ratio of the proposed genetic model (Table 3). To further investigate the genetics of glyphosate resistance, ten additional reciprocal families were created from the  $RS_2 \times P_0^S$  crosses. Family #1 in the  $P_0^S$  to  $RS_2$  cross displayed an above expected number of resistant individuals which resulted in a non-Mendelian phenotypic ratio ( $\chi^2 = 6.73$ ,  $P = 0.03$ ) (Table 4). Regardless, the combined GOF analysis for the  $RS_2 \times P_0^S$  cross converged to the expected 1:2:1 ( $F_2$ ) and 1:1 (back-cross) ratios for the proposed genetic model (Table 4).

The incompletely-dominant model predicted that the heterozygous genotype would display an intermediate phenotype compared to both parents. This was confirmed by the prevalence of IR phenotypes in the heterozygous  $F_1$  population that arose from crosses between the near-homozygous  $RS_2$  and  $P_0^P$  or  $P_0^S$  parents (Table 2). Furthermore, the  $F_1$  population demonstrated an intermediate  $GR_{50}$ , mortality, visual injury, and shikimic acid levels when contrasted to both resistant and susceptible parents (Table 1; Figure 1). Glyphosate resistance in another *C. canadensis* population was apparently conferred by a single, dominant nuclear gene (Montgomery et al. 2003). Our results clearly demonstrate an intermediate response to glyphosate of the heterozygous  $F_1$  and thus confirm suitability of the incompletely dominant model for the Houston, DE *C. canadensis* populations. Since dominant and incompletely-dominant models have been proposed for the inheritance of glyphosate resistance in *C. canadensis*, two distinct mechanisms of resistance may exist. Other investigations focus on elucidating the mechanism(s) of glyphosate resistance in *C. canadensis* and would certainly provide evidence as to the identity of the gene responsible for the resistant trait.

## Discussion

### Genetics of glyphosate resistance: evidence in transgenic crops

Glyphosate resistance was first engineered in tobacco (*Nicotiana tabacum* L.) through transformation with an insensitive *EPSPS* from *Salmonella typhimurium* that had a C<sup>327</sup> to T nucleotide transition, resulting in a prolyl<sup>101</sup> to seryl point mutation (GenBank M10947) (Comai et al. 1985). Transgenic glyphosate resistant petunia [*Petunia × hybrida* (Hook.) Vilm.] was also generated by *EPSPS* overexpression stemming from a 20-fold gene amplification (Shah et al. 1986) and by transformation with an insensitive *EPSPS* cloned from *Escherichia coli* that had a glycyl<sup>96</sup> to alanyl substitution (GenBank X00557) (Padgett et al. 1991). Kinetically, the mutated *EPSPS* had elevated apparent dissociation ( $K_i^{\text{glyphosate}}$ ) and Michaelis–Menten ( $K_m^{\text{PEP}}$ ) constants, suggesting lower enzyme efficiency compared to the wild-type *EPSPS* (Padgett et al. 1991). Most commercial transgenic crops, however, contain the *EPSPS* transgene cloned from *Agrobacterium* spp. CP4 (*CP4-EPSPS*). Since *CP4-EPSPS* is kinetically more efficient and shares less than 50% sequence homology to *EPSPS* from *E. coli* or *S. typhimurium* (class I), the enzyme is considered a class II *EPSPS* (He et al. 2003). Some engineered crops contain a second transgene, in addition to *CP4-EPSPS*, coding for a peptide that oxidizes the N–C<sub>α</sub> bond in glyphosate and yields α-aminomethylphosphonic acid (AMPA) and glycolic acid, two generally less phytotoxic metabolites (Barry et al. 1992). Both *CP4-EPSPS* and *GOX* transgenes expressed in crops were modified by mutagenesis, characterized by selection methods, and fused at 5'–translated region to a chloroplast transit sequence (CTS) and the 35S cauliflower mosaic virus promoter (CMV–35S) and at the 3'–nontranslated region, to the nopaline synthase (NOS) terminator sequence (Barry et al. 1992).

The first commercially available glyphosate resistant crops, soybean and cotton (*Gossypium hirsutum* L.), were transformed with the single *CP4-EPSPS* event. Molecular characterization elucidated that the transgenic soybean contained a single *CP4-EPSPS* insertion accompanied by fragments of the CTS, CMV–35S, and NOS sequences (Padgett et al. 1995). The event inherited glyphosate resistance in soybeans obeying Mendelian genetics as a single–dominant functional locus (Padgett et al. 1995). Similarly, transgenic cotton

with single or dual *CP4-EPSPS* insertions in a single locus segregated in ratios consistent with the monogenic dominant model (Nida et al. 1996). Concomitantly, transgenic wheat (*Triticum aestivum* L.) with the dual *CP4-EPSPS* and *GOX* event co-segregated in phenotypic proportions consistent with the single gene dominant model (Zhou et al. 1995). Maternal inheritance of glyphosate resistance has also been confirmed when *CP4-EPSPS* is engineered in the chloroplast genome (Ye et al. 2001). Overall, these results demonstrate that glyphosate resistance *in planta* can be conferred by monogenic or polygenic models of gene(s) encoded either in the cytoplasm or nucleus of cells.

### Genetics of evolved glyphosate resistance in plants

Approximately 300 herbicide resistant weed biotypes have been confirmed to date; however, only in less than 10% of the confirmed cases the mechanism and genetics of resistance were conclusively elucidated (Heap 2004). Herbicide resistance in the majority of characterized cases is conferred by a single, nuclear-encoded allele inherited as a dominant or incompletely-dominant trait (Gasquez 1997). Examples of recessive inheritance include resistance of several grasses to dinitroaniline herbicides (Wang et al. 1996; Zeng and Baird 1999). In the case of maternal effects, inheritance was only categorically demonstrated in resistance to triazines (Jasieniuk et al. 1996). Examples of more complex genetics include reports in wild oats (*Avena fatua* L.) of dominant diclofop resistance at low rates and reversal, dominant susceptibility, at high rates of the herbicide (Seefeldt et al. 1998). In another *A. fatua* example, triallate resistance was governed by two unlinked recessive alleles and inheritance was apparently maternal only at high triallate rates (Kern et al. 2002). Examples of polygenic resistance comprise the description of two independent nuclear alleles conferring fenoxaprop-P-ethyl resistance in blackgrass (*Alopecurus myosuroides* Huds.); identity of the resistance genes was ascribed to a mutant Acetyl-CoA carboxylase (EC 6.4.1.2) and a cytochrome P-450 mono-oxygenase (Letouzé and Gasquez 2001). More complex scenarios include additive gene effects in multiple resistant weeds where several alleles modulate the overall level of resistance (Preston 2003).

Evolved glyphosate resistance was first confirmed in two independent *L. rigidum* populations of Orange, New South Wales and Echuca, Northern Victoria, Australia (Powles

et al. 1998; Pratley et al. 1999). Genetic analysis of the Orange *L. rigidum* population revealed that glyphosate resistance was conferred by a single, incompletely-dominant allele under nuclear control (Lorraine-Colwill et al. 2001); to date however, the identity of the resistance gene remains elusive. Initial investigations found no indication that metabolism, uptake, or translocation mechanisms were involved in glyphosate resistance, nor did differences in EPSPS and 3-deoxy-7-phosphoheptulonate (DAHP; EC 2.5.1.54) synthase activities or *EPSPS* expression (Feng et al. 1999; Lorraine-Colwill et al. 1999). A more robust investigation found no evidence of *EPSPS* amplification or co-segregation of specific *EPSPS* isoforms with resistance; however, mRNA levels and EPSPS specific activity were higher in resistant plants (Baerson et al. 2002a). Enhanced *EPSPS* mRNA levels and endogenous activity of the enzyme, in addition to possible post-translational regulation of EPSPS, were also cited as resistant mechanisms in a glyphosate resistant Chinese foldwing [*Dicliptera chinensis* (L.) Juss.] population (Yuan et al. 2002). More recently, evidence was put forward that glyphosate resistance in *L. rigidum* was mediated by differences in the cellular transport of the herbicide (Lorraine-Colwill et al. 2003). Glyphosate import into plant cells is apparently ATP-driven by a phosphate transporter in the plasmalemma (Hetherington et al. 1998). Mutations in phosphate transporters significantly diminish movement of inorganic phosphate within plants and thus potentially the translocation of glyphosate (Versaw and Harrison 2002). Analogously, a mutant phosphate transporter in resistant plants could reduce glyphosate cellular transport and explain the proposed mechanism and genetic model for *L. rigidum*.

While evolved glyphosate resistance in *E. indica* was attributed to a C<sup>875</sup> to T transition coding for an insensitive prolyl<sup>101</sup> to seryl EPSPS isoform (Baerson et al. 2002b), no genetic analysis was conducted to validate the proposed single mechanistic model. A transversion at this same site, C<sup>875</sup> to A, codes for a threonyl<sup>101</sup> EPSPS isoform that is apparently also insensitive to glyphosate (Ng et al. 2004). In addition, a glyphosate resistant population from Lenggeng, Malaysia possessed an *EPSPS* sequence identical to the susceptible biotype, suggesting that at least another mechanism is capable of conferring glyphosate resistance in *E. indica* (Ng et al. 2004). In triangle waterfern [*Ceratopteris richardii* (L.) Brongn.], glyphosate resistance is governed by the independent nuclear *glt1* and *glt2* loci that are

inherited as incompletely-dominant or recessive traits, respectively (Chun and Hickok 1992). Results from these two species entertain the possibility that two or more mechanisms may modulate survival to glyphosate in some cases of evolved resistance. This assertion is evidenced in field bindweed (*Convolvulus arvensis* L.), where several concerted mechanisms at the cellular and metabolic levels may affect the level of glyphosate resistance (Westwood and Weller 1997). Concurrently, it was demonstrated that inheritance of glyphosate resistance within *C. arvensis* biotypes is the result of maternal effects and additive gene actions (Duncan and Weller 1987). Quantitative genetics of glyphosate resistance was also cited in maize (*Zea mays* L.) somaclones (Racchi et al. 1997). In the event that inheritance of glyphosate resistance is polygenic, weak selection pressure from sublethal applications and recombination through several generations may be necessary to increase resistant allele frequencies and select for the highest level of resistance. Mitigation of evolved polygenic resistance was proposed by periodically alternating sublethal herbicide applications with high rates of the herbicide, in addition to alternative control strategies (Gardner et al. 1998).

#### **Impact of genetics on *C. canadensis* resistance management**

The proposed partially-dominant model for inheritance of glyphosate resistance in *C. canadensis* contends that unless a fitness penalty is associated with the resistance trait, the *R*-allele should reside in the environment. In addition, expression of the *R*-allele in the heterozygous genotype ( $F_1$ ) estimated a  $GR_{50}$  of 1.21 kg glyphosate  $ha^{-1}$  (Table 1), which is well above the 0.85 kg  $ha^{-1}$  rate recommended by the manufacturer. Hence, under field conditions both homozygous and heterozygous genotypes should behave as a dominant trait. Finally, data from the reciprocal crosses confirmed that *C. canadensis* is essentially autogamous and self-compatible (Table 2). These combined statements would predict a rapid increase of resistant individuals within *C. canadensis* populations under continuous glyphosate selection. Not surprisingly, resistance in the Houston, DE populations evolved just after three years of continuous glyphosate selection (VanGessel 2001). Considering that glyphosate resistance has evolved in at least ten independent *C. canadensis* populations (Heap 2004), we suggest that enough genetic variability exists in *Coryza* for resistance to evolve rapidly.

Glyphosate resistance in *C. canadensis* is pollen borne (Table 3; Table 4). Evidence of entomophilous interactions have been cited in *Conyza* (Weaver 2001), entertaining the possibility of resistance transfer to adjacent *C. canadensis* populations. Furthermore, the anemochory nature of *C. canadensis* allows for achene dispersal to a maximum of 30 m in 16 km h<sup>-1</sup> wind (Dauer et al. 2003). This effective dispersal mechanism combined with *C. canadensis* potential to produce 240,000 achenes per growing season (Muenscher 1935), would certainly facilitate resistance spread to adjacent areas. Containment of evolved glyphosate resistance may require the use of an integrated management approach. For example, mechanical control and a combination of pre-emergence and residual herbicides provide effective *C. canadensis* management (Brown and Whitwell 1988; VanGessel et al. 2001). Farmers should not only contemplate the economics associated with weed management, but rather focus on adopting effective and long-term strategies that will preserve the sustainability of current production systems.

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**Table 1.** Summary of whole-plant rate responses for the evaluated *Conyza canadensis* populations. Numbers in parenthesis designate the 95% confidence intervals ( $GR_{50}$ ;  $I_{50}$ ) or 95% fiducial limits ( $LD_{50}$ ) for the preceding estimated parameter. Spearman's correlation ( $r^2$ ) estimated the association strength between shikimic acid levels and biomass or visual injury.

Population <sup>a</sup>	Source	$GR_{50}$ <sup>b</sup>	$LD_{50}$ <sup>c</sup>	$I_{50}$ <sup>d</sup>	biomass ( $r^2$ ) <sup>e</sup>	injury ( $r^2$ ) <sup>e</sup>
P <sub>0</sub> <sup>P</sup>	Ames, IA	0.53 (0.43–0.63)	0.92 (0.59–1.37)	1.88 (1.20–2.57)	–0.64	0.75
P <sub>0</sub> <sup>S</sup>	Georgetown, DE	0.50 (0.41–0.59)	1.19 (0.75–1.81)	2.06 (1.44–2.67)	–0.60	0.74
P <sub>0</sub> <sup>R</sup>	Houston, DE	2.11 (1.43–2.79)	8.77 (6.17–13.73)	3.87 (0.95–6.80)	–0.73	0.82
RS <sub>1</sub>	Houston, DE	2.00 (1.37–2.63)	9.73 (6.59–16.77)	4.38 (0.18–8.59)	–0.68	0.84
RS <sub>2</sub>	Houston, DE	2.03 (1.36–2.70)	10.59 (7.04–16.88)	3.10 (1.27–4.94)	–0.67	0.76
F <sub>1</sub> <sup>f</sup>	Artificial cross	1.21 (0.85–1.57)	2.85 (1.80–4.58)	2.33 (1.48–3.19)	–0.79	0.85
F <sub>2</sub> <sup>f</sup>	Artificial cross	1.62 (1.11–2.14)	3.76 (2.32–6.44)	3.38 (1.17–5.59)	–0.69	0.70

<sup>a</sup> = P<sub>0</sub><sup>P</sup>, pristine population; P<sub>0</sub><sup>S</sup>, glyphosate susceptible population; P<sub>0</sub><sup>R</sup>, glyphosate resistant population; RS<sub>1</sub>, P<sub>0</sub><sup>R</sup> selected at 2.0 kg glyphosate ha<sup>–1</sup>; RS<sub>2</sub>, RS<sub>1</sub> selected at 2.0 kg glyphosate ha<sup>–1</sup>; F<sub>1</sub>, first filial generation; F<sub>2</sub>, second filial generation.

<sup>b</sup> = glyphosate rate in kg ha<sup>–1</sup> that reduced biomass accumulation by 50%.

<sup>c</sup> = glyphosate rate in kg ha<sup>–1</sup> that inflicted 50% mortality in the population.

<sup>d</sup> = glyphosate rate in  $\text{kg ha}^{-1}$  that resulted in accumulation of half of the total extractable shikimic acid in the tissue of treated plants.

<sup>e</sup> = the probability of  $|r|$  was  $>$  than 0.001 for all estimates, therefore the null hypothesis ( $H_0$ ) that  $r = 0$  was rejected.

<sup>f</sup> = three randomly selected rosettes per each of the 20 generated families ( $n = 60$ ) were used to test the rate response to glyphosate.

**Table 2.** Estimates of emasculation efficiency (EE) and cross pollination (allogamy) between *Conyza canadensis* from Ames, IA ( $P_0^P$ ), Georgetown, DE ( $P_0^S$ ) and Houston, DE ( $P_0^R$ ) selected twice at 2.0 kg glyphosate ha<sup>-1</sup> (RS<sub>2</sub>). EE represented the percent germinated achenes (G) from the total estimated achenes emasculated (AE). Percent allogamy (PA) and percent compatibility (PC) were estimated from the frequency of glyphosate resistant (R), intermediate-resistant (IR), and susceptible (S) first filial (F<sub>1</sub>) descendants within each family.

Family	Emasculated <sup>a</sup>			Assisted cross <sup>b</sup>						Artificial cross <sup>c</sup>						g
	RS <sub>2</sub>			RS <sub>2</sub> to P <sub>0</sub> <sup>P</sup>			P <sub>0</sub> <sup>P</sup> to RS <sub>2</sub>			RS <sub>2</sub> to P <sub>0</sub> <sup>P</sup>			P <sub>0</sub> <sup>P</sup> to RS <sub>2</sub>			
	AE	G	EE <sup>d</sup>	S <sup>e</sup>	IR	PA <sup>f</sup>	IR	R	PA	S	IR	PC <sup>f</sup>	IR	R	PC	
1	280	2	99.3	24	4	14.3	3	25	10.7	1	31	96.9	28	1	96.6	g
2	350	4	98.9	24	2	7.7	0	25	0.0	0	25	100.0	22	0	100.0	
3	245	0	100.0	25	0	0.0	2	24	7.7	0	29	100.0	26	0	100.0	
4	245	2	99.2	33	4	10.8	1	31	3.1	2	30	93.8	27	0	100.0	
5	315	0	100.0	32	4	11.1	2	34	5.6	1	24	96.0	32	0	100.0	
RS <sub>2</sub>			RS <sub>2</sub> to P <sub>0</sub> <sup>S</sup>			P <sub>0</sub> <sup>S</sup> to RS <sub>2</sub>			RS <sub>2</sub> to P <sub>0</sub> <sup>S</sup>			P <sub>0</sub> <sup>S</sup> to RS <sub>2</sub>				
1	280	1	99.6	27	0	0.0	0	25	0.0	0	28	100.0	25	1	96.2	

2	350	4	98.9	31	4	11.4	3	26	10.3	0	25	100.0	31	1	96.9
3	350	3	99.2	24	3	11.1	0	28	0.0	2	32	94.1	25	1	96.2
4	315	0	100.0	25	2	7.4	2	27	6.9	1	27	96.4	24	0	100.0
5	350	3	99.2	29	5	14.7	3	26	10.3	2	26	92.9	29	0	100.0
Total	3080	19	99.4	274	28	9.3	16	271	5.6	9	277	96.9	269	4	98.5

<sup>a</sup> = yellow perfect florets were manually excised from the capitula pre-anthesis and the white pistillate florets allowed to mature inside a DelNet<sup>®</sup> bag.

<sup>b</sup> = at anthesis intact RS<sub>2</sub> and P<sub>0</sub><sup>S</sup> inflorescences were covered with a DelNet<sup>®</sup> bag and florets permitted to cross-pollinate.

<sup>c</sup> = the receptor capitula were emasculated pre-anthesis and the remaining pistillate florets were fertilized with intact capitula from the pollen donor plant.

<sup>d</sup> = emasculation efficiency (EE) represented the percent non-germinated achenes from the total estimated achenes evaluated.

<sup>e</sup> = susceptible (S) comprised 10 cm diameter rosettes killed at 2.0 kg glyphosate ha<sup>-1</sup>. Per contra, resistant (R) and intermediate-resistant (IR) represented rosettes that reached reproductive stage and demonstrated ≤ 30% and 31–69% visual injuries at the same glyphosate rate and phenological stage, respectively.

<sup>f</sup> = percent allogamy (PA) or percent compatibility (PC) represented the proportion IR phenotypes within the total rosettes treated.



**Table 3.** *R*-allele segregation in ten second filial ( $F_2$ ) families generated by artificial crosses between the pristine *Conyza canadensis* from Ames, IA ( $P_0^P$ ) and the Houston, DE population ( $P_0^R$ ) selected twice at 2.0 kg glyphosate ha<sup>-1</sup> ( $RS_2$ ). An  $F_2$  family originated from a single first filial ( $F_1$ ) *C. canadensis* plant allowed to self-pollinate in isolation. For the back-crosses, the  $F_1$  served as the pollen donor to a previously emasculated  $RS_2$  ( $BC_r$ ) or  $P_0^P$  ( $BC_p$ ) pollen receptor plant that arose from an achene of the original  $RS_2$  or  $P_0^P$  parent.

Origin of $F_1$ Parents <sup>a</sup>			Observed phenotype <sup>b</sup>				Expected <sup>c</sup>		
Donor	Receptor	$F_2$ family No.	R	IR	S	Total	R : IR : S	$\chi^2$	$P > \chi^2$
$RS_2$	$P_0^P$	1	5	13	11	29	7.25 : 14.5 : 7.25	2.79	0.25
		2	10	15	6	31	7.75 : 15.5 : 7.75	1.06	0.59
		3	6	16	11	33	8.25 : 16.5 : 8.25	1.54	0.46
		4	5	16	5	26	6.5 : 13 : 6.5	1.38	0.50
		5	6	11	8	25	6.25 : 12.5 : 6.25	0.68	0.71
		Total	32	71	41	144	36 : 72 : 36	1.15	0.56
$P_0^P$	$RS_2$	1	7	17	6	30	7.5 : 15 : 7.5	0.60	0.74

	2	9	15	7	31	7.75 : 15.5 : 7.75	0.29	0.86
	3	4	17	7	28	7 : 14 : 7	1.93	0.38
	4	12	12	5	29	7.25 : 14.5 : 7.25	4.24	0.12
	5	5	14	11	30	7.5 : 15 : 7.5	2.53	0.28
	Total	37	75	36	148	37 : 74 : 37	0.04	0.98
<hr/>								
	Combined F <sub>2</sub> families <sup>d</sup>	69	146	77	292	73 : 146 : 73	0.44	0.80
	Combined BC <sub>r</sub> families <sup>d</sup>	62	55	---	117	58.5 : 58.5 : 0	0.42	0.52
	Combined BC <sub>p</sub> families <sup>d</sup>	---	41	47	88	0 : 44 : 44	0.41	0.52
<hr/>								
Performance of	P <sub>0</sub> <sup>P</sup>	0	0	59	59	0 : 0 : 59	---	---
parents	P <sub>0</sub> <sup>R</sup>	78	0	4	82	82 : 0 : 0	---	---
	RS <sub>1</sub>	79	0	0	79	79 : 0 : 0	---	---
	RS <sub>2</sub>	84	0	0	84	84 : 0 : 0	---	---
<hr/>								

<sup>a</sup> = first filial families ( $F_1$ ) were produced by reciprocal intraspecific artificial crosses between the  $RS_2$  and  $P_0^P$  parents. Donor represented the pollen donor *Conyza canadensis* parent with intact capitula. Receptor was the *C. canadensis* parent with pistillate florets (emasculated) that accepted the pollen.

<sup>b</sup> = observed resistant (R), intermediate-resistant (IR), and susceptible (S) phenotypes in the progeny of a single  $F_1$  per family allowed to self-pollinate ( $F_2$ ). Twenty DAT of 2.0 kg glyphosate  $ha^{-1}$ , R, IR, and S phenotypes comprised rosettes with  $\leq 30\%$ , 31–69 %, and  $\geq 70\%$  visual herbicide injury, respectively. Only S individuals failed to reach reproductive stage. All plants were treated at the 10 cm diameter rosette stage.

<sup>c</sup> = expected Mendelian R, IR, and S segregation ratios for the incompletely-dominant single gene model (1:2:1).

<sup>d</sup> = the homogeneity  $\chi^2$  test among families was non-significant, therefore data were combined for the  $\chi^2$  goodness-of-fit test. Combined  $F_2$  families,  $\chi^2 = 1.77$ ,  $P = 0.99$ ; combined  $BC_r$  families,  $\chi^2 = 4.79$ ,  $P = 0.85$ ; combined  $BC_s$  families,  $\chi^2 = 7.68$ ,  $P = 0.57$ .

**Table 4.** *R*-allele segregation in ten second filial ( $F_2$ ) families generated by artificial crosses between the susceptible *Conyza canadensis* from Georgetown, DE ( $P_0^S$ ) and the Houston, DE population ( $P_0^R$ ) selected twice at 2.0 kg glyphosate ha<sup>-1</sup> ( $RS_2$ ). An  $F_2$  family originated from a single first filial ( $F_1$ ) *C. canadensis* plant allowed to self-pollinate in isolation. For the back-crosses, the  $F_1$  served as the pollen donor to a previously emasculated  $RS_2$  ( $BC_r$ ) or  $P_0^S$  ( $BC_s$ ) pollen receptor plant that arose from an achene of the original  $RS_2$  or  $P_0^S$  parent.

Origin of $F_1$ Parents <sup>a</sup>			Observed phenotype <sup>b</sup>				Expected <sup>c</sup>		
Donor	Receptor	$F_2$ family No.	R	IR	S	Total	R : IR : S	$\chi^2$	$P > \chi^2$
$RS_2$	$P_0^S$	1	6	22	8	36	9 : 18 : 9	2.00	0.37
		2	12	13	6	31	7.75 : 15.5 : 7.75	3.13	0.21
		3	9	13	12	34	8.5 : 17 : 8.5	2.41	0.30
		4	5	19	5	29	7.25 : 14.5 : 7.25	2.79	0.25
		5	6	15	14	35	8.75 : 17.5 : 8.75	4.37	0.11
		Total	38	82	45	165	41.25 : 82.5 : 41.25	0.60	0.74
$P_0^S$	$RS_2$	1	16	15	6	37	9.25 : 18.5 : 9.25	6.73	0.03

	2	11	14	5	30	7.5 : 15 : 7.5	2.53	0.28
	3	7	10	9	26	6.5 : 13 : 6.5	1.69	0.43
	4	9	14	8	31	7.75 : 15.5 : 7.75	0.36	0.84
	5	5	20	5	30	7.5 : 15 : 7.5	3.33	0.19
	Total	48	73	33	154	38.5 : 77 : 38.5	3.34	0.19
<hr/>								
	Combined F <sub>2</sub> families <sup>d</sup>	86	155	78	319	79.75 : 159.5 : 79.75	0.65	0.72
	Combined BC <sub>r</sub> families <sup>d</sup>	57	54	---	111	55.5 : 55.5 : 0	0.08	0.78
	Combined BC <sub>s</sub> families <sup>d</sup>	---	46	53	99	0 : 49.5 : 49.5	0.49	0.48
<hr/>								
Performance of parents	P <sub>0</sub> <sup>s</sup>	0	0	73	73	0 : 0 : 73	---	---
	RS <sub>2</sub>	84	0	0	84	84 : 0 : 0	---	---

<sup>a</sup> = first filial families (F<sub>1</sub>) were produced by reciprocal intraspecific artificial crosses between the RS<sub>2</sub> and P<sub>0</sub><sup>s</sup> parents. Donor represented the pollen donor *Conyza canadensis* parent with intact capitula. Receptor was the *C. canadensis* parent with pistillate florets (emasculated) that accepted the pollen.

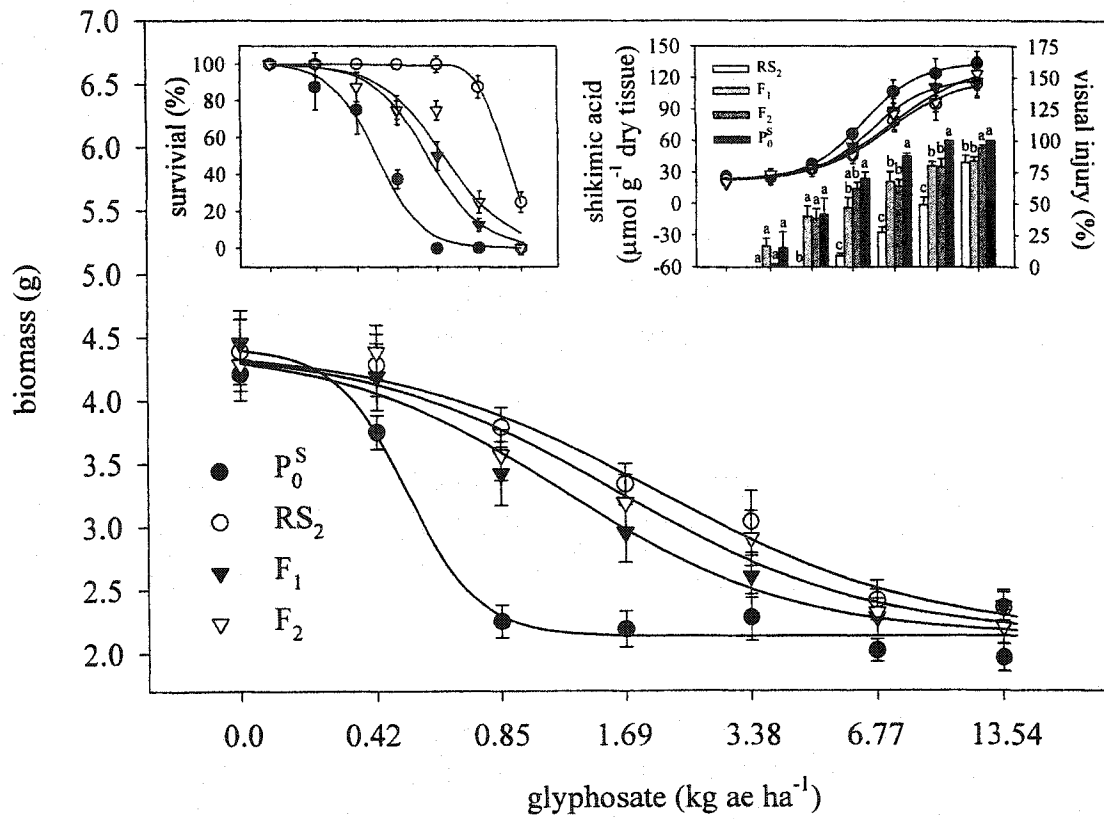
<sup>b</sup> = observed resistant (R), intermediate-resistant (IR), and susceptible (S) phenotypes in the progeny of a single F<sub>1</sub> per family allowed to self-pollinate (F<sub>2</sub>). Twenty DAT of 2.0 kg glyphosate ha<sup>-1</sup>, R, IR, and S phenotypes comprised rosettes with ≤ 30%,

31–69 %, and  $\geq 70\%$  visual herbicide injury, respectively. Only S individuals failed to reach reproductive stage. All plants were treated at the 10 cm diameter rosette stage.

<sup>c</sup> = expected Mendelian R, IR, and S segregation ratios for the incompletely–dominant single gene model (1:2:1).

<sup>d</sup> = the homogeneity  $\chi^2$  test among families was non–significant, therefore data were combined for the  $\chi^2$  goodness–of–fit test.

Combined F<sub>2</sub> families,  $\chi^2 = 3.41$ ,  $P = 0.95$ ; combined BC<sub>r</sub> families,  $\chi^2 = 3.68$ ,  $P = 0.93$ ; combined BC<sub>s</sub> families,  $\chi^2 = 3.32$ ,  $P = 0.95$ .



**Figure 1.** Main plot: rate response at 20 DAT of *Conyza canadensis* from Georgetown, DE ( $P_0^S$ ; ●; black bar), the Houston, DE selected twice at 2.0 kg glyphosate ha<sup>-1</sup> (RS<sub>2</sub>; ○; white bar), the first filial (F<sub>1</sub>; ▼; gray bar), and the second filial (F<sub>2</sub>; ▽; dark gray bar) populations to glyphosate. Left insert: survival of *C. canadensis* to glyphosate; lines represent the mortality estimated by PROBIT. Right insert: endogenous shikimic acid levels (symbols) and visual herbicide injury (bars) of treated plants. Letters above bars designate the minimum statistical difference (LSD<sub>α=0.05</sub>) between populations for a single rate. The x axes in inserts correspond to the glyphosate rates described in the main plot. For all graphs, each data point or bar represents the mean four replications and two experiments conducted at different times ( $n = 8$ ). F<sub>1</sub> and F<sub>2</sub> rate responses were conducted on a population comprised of three randomly selected plants per each of the 20 generated families ( $n = 60$ ). Extensions on symbols or bars designate the standard error associated with individual means ( $\sigma_M$ ).

## CHAPTER 4. FIRST CONFIRMED HYBRID IN THE AMERICAN *CONYZA*: IMPLICATIONS FOR TRANSFER OF GLYPHOSATE RESISTANCE

A paper submitted to *Theoretical and Applied Genetics*<sup>1</sup>

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### Abstract

Hybrids were isolated from crosses between the diploids dwarf fleabane (*Conyza ramosissima* Cronq.;  $2n = 18$ ) and the glyphosate [*N*-(phosphonomethyl) glycine] resistant horseweed (*C. canadensis* (L.) Cronq.;  $2n = 18$ ). Hybridization in reciprocal assisted crosses ranged from 0% to 9% within families and > 95% was estimated in the artificial *C. canadensis* to *C. ramosissima* unidirectional cross. Multivariate quantitative trait analysis suggested that the interspecific hybrid ( $F_1^H$ ) shared an intermediate phenotype between both parents with more similarity to *C. ramosissima*. In addition, the  $F_1^H$  was stable, evidenced heterosis, and possessed negligible postzygotic reproductive barriers that confirmed fertility of the hybrid taxon. Inheritance of glyphosate resistance in *C. canadensis* followed the hybrid resistance model in the  $F_1^H$  and the nuclear encoded, incompletely-dominant single gene (*R*-allele) model in the hybrid progeny ( $F_2^H$ ). Furthermore, transgressive segregation was observed in most  $F_2^H$  morphological traits. Backcrosses confirmed that the *R*-allele introgressed between both parental through the  $F_1^H$ . We argue that the hybrid taxon likely formed without an increase in ploidy (homoploid) and that adequate fitness and niche differentiation are prerequisites for successful adaptation in the environment. Hybridization

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in *Conyza* may thus complicate the containment of glyphosate resistance in current agroecosystems.

### Introduction

Asteraceae embody one of the largest and most diverse families within dicotyledonous plants, members of which are present in every global environment except for aquatic habitats (Cronquist 1980). The approximate 1,100 genera comprising the family are characterized by composite flowers enveloped by phyllaries and clustered in an involucre pseudanthium that form the capitulum. The genus *Conyza* Less., represented by approximately 60 species, is comprised of annual herbaceous plants that prosper chiefly in tropical and subtropical regions of the globe (Nesom 1990). Species endemic to the United States of America (US) include the winter or summer annual forbs dwarf fleabane (*Conyza ramosissima* Cronq.) and horseweed [*C. canadensis* (L.) Cronq.] (Cronquist 1980). *C. ramosissima* was originally described in Illinois as *Erigeron divaricatus* (Michaux 1803). The species was later annexed to the genus *Conyza* based on the eligulate character of its multiseriate pistillate florets (Cronquist 1980). Both *C. ramosissima* and *C. canadensis* are ubiquitous to disturbed habitats and play important roles in primary ecological successions; however, only *C. canadensis* is reported to reduce yields in row crops, serve as an alternate host for diverse pests, and limit grazing by reducing the palatability of pastures (Steyermark 1963; Regehr and Bazzaz 1979). *C. ramosissima* is found from North Dakota to Pennsylvania and south from New Mexico to Alabama in the US, in southern Canada, and northern Mexico, while the more cosmopolitan *C. canadensis* is distributed throughout the Americas, West Indies, Europe, and Africa (Steyermark 1963). Interestingly, the *Conyza* taxon represents the most successful case of intercontinental colonization of the Americas to Old World, to the extent that *C. canadensis* and tall fleabane (*C. floribunda* H.B.K.) are probably the most widely distributed species throughout the world (Thébaud and Abbott 1995).

Interspecific hybridization refers to the cross-fertilization between two species that procreates a novel fertile or infecund progeny sharing phenotypic traits of both parents; this process of interspecific gene transfer is crucial in promoting genetic diversity and genome

evolution (Abbott 1992; Barton 2001). While interspecific nuclear gene transfer can occur between phylogenetically related species, recent evidence suggests that at least some mitochondrial genes are frequently introgressed between distantly related angiosperms (Bergthorsson et al. 2003). Pragmatically, breeding efforts often rely on interspecific hybridization for plant improvement and the generation of novel crop species (Anamthawat-Jónsson 2001; Repellin et al. 2001). However, this natural process may also hinder crop production and weed management as interspecific transfer of herbicide resistance and genetically engineered genes has been documented (Chèvre et al. 2000; Franssen et al. 2001). Furthermore, interspecific hybridization may facilitate the rapid evolution and adaptation of introduced plant pathogens and contribute to the genetic diversity of pathogenic fungi, nematodes, and insects (Teal and Oostendorp 1995; Van der Beek and Karssen 1997; Schardl and Craven 2003). It is accepted that hybridization contributes significantly to the evolution of novel taxa either as intraspecific taxa or stable homoploid or allopolyploid introgressants, provided niche differentiation and appreciable hybrid fertility exists. In the absence of fertility, allopolyploid hybrids can reproduce asexually (Abbott 1992). A recent case study of 171 hybrid populations suggested that transgressive segregation of complementary genes is a major contributor to niche differentiation and thus contributed significantly to the rapid adaptation of hybrid lineages (Rieseberg et al. 1999).

Glyphosate [*N*-(phosphonomethyl) glycine] resistant crops provide farmers with a simple, economical, and effective tool to manage a diverse weed flora, hence favoring the rapid adoption of this technology in many US crop production systems. The mechanism of glyphosate action comprises the competitive inhibition with respect to the phosphate moiety of phosphoenolpyruvate in the reaction mediated by 3-phosphoshikimate 1-carboxyvinyltransferase (EPSPS; EC 2.5.1.19) (Steinrücken and Amrhein 1980). Glyphosate resistance *in planta* generally evolves at a low frequency compared to other herbicides. At present, six species resistant to EPSPS-inhibiting herbicides have been reported, including confirmation in nine independent *C. canadensis* populations within the US (Heap 2004). The fact that several *Conyza* taxa coexist in analogous ecological niches raised a question about the potential for interspecific gene transfer within the genus. Herein we report on the potential for introgressive hybridization (introgression) between *C. canadensis* and *C.*

*ramosissima*, assess postzygotic reproductive barriers of the interspecific hybrid ( $F_1^H$ ) populations, and propose a genetic model for the inheritance of the gene associated with glyphosate resistance [*R*-allele].

## Materials and Methods

### Source of plant materials

The glyphosate susceptible (S) *C. ramosissima* specimens were collected prior to anthesis from the Ontario Cemetery, in Ames, IA, in June 2003; according to the City of Ames, no glyphosate was used in the Cemetery premises. The *C. canadensis* population resistant (R) to glyphosate was collected near Houston, DE and evolved in a no-tillage soybean field that received from 1999 to 2000, one or two seasonal applications of 1.6 kg acid equivalents (ae) isopropylamine salt of glyphosate  $\text{ha}^{-1}$  (VanGessel 2001). Complete specimen sets of the parental and hybrid progenies were deposited at the Ada Hayden Herbarium (ISC; accession No. 435636–435644). Duplicate specimens sets were also deposited at the Botanical Research Institute of Texas (BRIT) and the New York Botanical Garden (NY).

### Growth conditions

*C. ramosissima* specimens collected from the field were transplanted into 12 cm diameter pots with a peat:perlite:loam (1:2:1) soil-mix media; conversely, *C. canadensis* plants originated from achenes that were germinated in flats containing soil-mix media and later transplanted to 12 cm diameter pots. All crosses were performed in growth cabinets set at a 16 hr photoperiod, 25:35 C diurnal:nocturnal, 70% to 90% relative humidity (RH), and  $600 \mu\text{mol}^{-2} \text{m s}^{-1}$  photosynthetic photon flux density (PPFD) conditions. The *C. canadensis* and *C. ramosissima* parental and sibling populations were grown in a greenhouse set to 25 to 35 C and 50 to 80% RH diurnal and 20 to 25 C and 50% RH nocturnal conditions; natural light was supplemented with 16 hr of  $600$  to  $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$  PPFD artificial illumination. Pots were irrigated as needed and fertilized (Miracle Gro Excel, Scott-Sierra Horticultural Products Co., 14111 Scottlawn Road, Marysville, OH 43041) one month after establishment.

## Performance of *Conyza* populations to glyphosate

### *Estimation of individuals resistant, intermediate-resistant, and susceptible within the populations*

The manufacturer's recommended rate for *Conyza* at the 10 cm diameter rosette stage is 0.85 kg ha<sup>-1</sup> glyphosate (Roundup UltraMAX™, Monsanto, 700 Chesterfield Parkway North, St. Louis, MO 63198) (Anonymous 2004). Concurrently, R and intermediate-resistant (IR) phenotypes were classified as rosettes at the 10 cm diameter stage that developed  $\leq 30\%$  and 31–69% visual injury 20 days after application of 2.0 kg glyphosate ha<sup>-1</sup>, respectively. Individuals demonstrating  $\geq 70\%$  visual injury and effectively controlled at this same rosette stage and glyphosate rate were defined as the S phenotype. The proportion of R, IR, and S individuals within the populations was estimated by classifying plants treated with 2.0 kg ha<sup>-1</sup> glyphosate to these parameters.

### *Whole-plant rate responses*

Plants were treated with deionized water (dH<sub>2</sub>O; control), 0.5, 1, 2, 4, 8, or 16 fold the recommended glyphosate rate for *Conyza* rosettes at the 10 cm diameter stage. Treatments were applied 30 cm above the canopy of plants through an 80015-E nozzle (TeeJet Spraying Systems, PO Box 7900, Wheaton, IL 60189–7900) in a CO<sub>2</sub>-powered spray chamber (SB5–66, DeVries Manufacturing, Route 1 Box 184, Hollandale, MN 56045) delivering 187 l ha<sup>-1</sup> at 2.8 kg per cm<sup>2</sup>. Spray coverage was confirmed visually and plants were allowed to dry prior transfer to the greenhouse. Twenty days post treatment, efficacy was estimated by visually examining the glyphosate-treated plants and contrasting the level of injury to the control; plants were graded on a percent scale considering 0% for non-injured and 100% for completely necrotic plants. Plants were then cut at the soil surface, placed in paper bags, and dried at 35 C for 48 h. Biomass was measured by weighing individual samples and used to estimate the glyphosate rate that inhibited plant growth by 50% ( $GR_{50}$ ) (refer to the Statistical analysis section).

### *Extraction and determination of endogenous shikimic acid*

Spectrophotometric quantification of shikimic acid (3*R*,4*S*,5*R* trihydroxy-1-cyclohexene-1-carboxylic acid) accumulation as a function of the rate of applied glyphosate was determined following a modified protocol of Cromartie and Polge (2002). In synopsis, 0.5 g of dry tissue from each rate-response sample was ground with 2.5 mm glass beads in a BeadBeater<sup>®</sup> (BioSpec Products, P.O. Box 788, Bartlesville, OK 74005-0788). Thereafter, shikimic acid was extracted for 48 h in a proportion of 1:10 tissue:0.25 N HCl. The supernatant, after centrifugation at 15,000 g, was derivatized with periodate and the shikimic acid chromophore stabilized with NaOH:Na<sub>2</sub>SO<sub>3</sub>, before spectrophotometric quantification at 382 nm. Samples were assayed in duplicate and standard curves prepared at a range of 1 to 60  $\mu\text{mol ml}^{-1}$  shikimic acid (Sigma-Aldrich, 3050 Spruce Street, Saint Louis, MO 63103).

### *Phenotypic confirmation of parents*

Greenhouse evaluations suggested that the initial *C. canadensis* population collected in the field was comprised of approximately 5% glyphosate S individuals (data not shown). Hence, a stable-homogeneous R population was isolated by undergoing two cycles of recurrent selection at 2.0 kg glyphosate ha<sup>-1</sup>. In addition, *C. canadensis* parents used in crosses were confirmed phenotypically by treating 10 cm diameter rosettes with a 2.0 kg ha<sup>-1</sup> rate and assessing plant survival 20 days post glyphosate treatment. *C. ramosissima* plants used in the parental crosses were not confirmed phenotypically as individuals were collected directly from the field. However, anecdotal information suggested that the *C. ramosissima* population evolved in the absence of exposure to glyphosate. Furthermore, rate response of plants derived from the original *C. ramosissima* population resulted in uniform and effective control at 0.85 kg glyphosate ha<sup>-1</sup>. Parents used in the backcrosses originated from achenes of plants used in the parental crosses. The backcross *C. canadensis* parents were confirmed as indicated in this section. In addition, phenotypic confirmation of backcross *C. ramosissima* parents was done by treating 10 cm diameter rosettes with 0.4 kg glyphosate ha<sup>-1</sup>. This sublethal rate caused  $\leq 60\%$  visual injury but did not kill plants, thus allowing for non-destructive identification of the S phenotype at the rosette stage.

## Interspecific hybridization

### *Assisted and artificial C. canadensis x C. ramosissima crosses*

The gynomonoecious *C. canadensis* and *C. ramosissima* possess white pistillate ray florets in the periphery and yellow perfect disk florets in the capitulum core. Pollen release may occur before the capitula unveil, thus both assisted or artificial crosses were conducted pre-anthesis. Assisted reciprocal crosses were performed by covering the inflorescence of *C. canadensis* and *C. ramosissima* plants with a PQ218 DelNet<sup>®</sup> bag (DelStar Technologies, 601 Industrial Drive, Middletown, DE 19709), hence permitting physical interaction among capitula and restricting pollen release within the bag. Artificial crosses were conducted by manually excising *C. ramosissima* disk florets with forceps under a magnifying lens (emasculatation). Upon stigma protrusion of the remaining pistillate florets, fertilization was accomplished by gently rubbing the intact capitula of donor *C. canadensis* plants. Non-emasculated capitula were removed from *C. ramosissima* plants to prevent self-pollination. Approximately 50 emasculated capitula per *C. ramosissima* plant were fertilized daily for one week and permitted to mature in isolation on the mother plant. Thereafter,  $F_1^H$  achenes were harvested from *C. ramosissima* plants when physiologically mature, germinated in flats containing soil-mix media, and seedlings individually transplanted to 12 cm pots;  $F_1^H$  plantlets were allowed to grow under the previously described greenhouse conditions.

The efficiency of emasculatation was tested by assessing the germinability of achenes in ten emasculated capitula per *C. ramosissima* plant that developed inside DelNet<sup>®</sup> bags in the absence of pollen. If emasculatation was completely effective, no viable achenes should be produced. Introgression of the *R*-allele was assessed with the assisted reciprocal and artificial unidirectional methods by crossing ten *C. canadensis* and *C. ramosissima* plant pairs (families) in isolation. Interspecific compatibility was estimated by backcrossing one  $F_1^H$  plant per family to individuals that originated from achenes of the original *C. canadensis* ( $BC_c^H$ ) and *C. ramosissima* ( $BC_r^H$ ) parents.

### Assessment of postzygotic reproductive barriers and characterization of hybrids

Achene viability was tested according to the Association of Official Seed Analysts (AOSA) standard germination procedure recommended for Asteraceae (AOSA 2003).

Hybrid inviability was assessed by germinating 100 achenes per  $F_1^H$  family in  $dH_2O$ -moistened blue blotter circles (Anchor Paper Co., 480 Broadway Street, St. Paul, MN 55101) inside plastic petri dishes in the previously described growth cabinet conditions. Dishes were monitored daily for two weeks and germinated achenes counted and removed; the remnant achenes were then classified as dormant or non-viable based on tetrazolium test results (Moore 1985). Experiments were repeated in time one week after the initial assessment. Achene viability of  $F_1^H$  plants allowed to self-pollinate ( $F_2^H$ ) provided an estimate of the level of hybrid sterility, while the viability of  $BC_c^H$  and  $BC_r^H$  achenes provided an estimate the level of hybrid breakdown.

Quantitative traits were determined on five samples from each of ten randomly selected plants per *C. canadensis* or *C. ramosissima* population, or one randomly selected plant per each of the ten  $F_1^H$  families. Determinations were performed two times ( $n = 100$ ). Rosette measurements were taken five to six weeks after emergence. These measurements included rosette diameter, rosette leaf number, leaf length, leaf width, leaf shape (leaf length  $\div$  leaf width), leaf dentation, adaxial leaf trichomes, leaf area, leaf weight, and the specific leaf area (SLA; leaf area  $\div$  leaf weight). Two weeks post stem differentiation but prior to anthesis, cauline measurements taken included leaf length, leaf width, leaf shape, leaf dentation, adaxial leaf trichomes, leaf area, leaf weight, and SLA; branch number per plant and stem diameter at 2 cm from the soil or axis of the rosette. Morphometric parameters were measured post-anthesis but prior to physiological maturity and included capitula length, capitula width, capitula shape, the number of pistillate and perfect florets, and total florets per capitulum. Achene length was recorded at maturity. Dimensional measurements were recorded with a digital caliper; area was estimated by outlining leaves on millimetric paper (2 mm divisions) and calculating the area therein. Trichome numbers were determined using a stereoscope by counting the pubescence of leaf disks (30 mm<sup>2</sup> area) excised with a hole puncher.

### Statistical analysis

The Statistical Analysis Software (SAS) was utilized to conduct all data analyses (SAS 2000). Replication in time was tested for patterns of covariance matrices that satisfied the Huynh–Feldt condition (option PRINTE) and if accepted,  $F$ -statistics assessed the univariate analyses for within time effects and related interactions (Huynh and Feldt 1970). Achene viability tests, arranged in a complete randomized design (CRD) with one replication in time, were subjected to analysis of variance (ANOVA; PROC GLM) as well as mean separation by Fisher's least significant difference ( $LSD_{\alpha=0.05}$ ) when ANOVA identified significant taxon effects. Whole-plant glyphosate rate responses were arranged in a randomized complete block (RCB) design with four replications and repeated once in time; biomass data were subjected to ANOVA and  $GR_{50}$  values estimated by PROC NLIN following the log-logistic model (Seefeldt et al. 1995). Similarly, shikimic acid content data were subjected to log-logistic analysis and the glyphosate rate that resulted in 50% accumulation of endogenous shikimic acid ( $I_{50}$ ) was determined accordingly. The overall model fit to the data was tested statistically by pseudo coefficients of determination [ $R^2_{(\text{pseudo})}$ ] and lack-of-fit (LOF) tests, and graphically by the distribution of residuals;  $P$ -values for the absolute difference between two estimated  $GR_{50}$  values ( $|\lambda_{50}|$ ) were calculated by reparameterization of the Brain–Cousens model (Seefeldt et al. 1995; Schabenberger et al. 1999). Binary transformation of visual injury data based on the classification for R ( $< 69\%$ ) and S ( $\geq 70\%$ ) phenotypes and analysis by a modified Newton–Raphson algorithm (PROC PROBIT) permitted estimation of the glyphosate rate that inflicted 50% mortality within the population ( $LD_{50}$ ) (Collett 2002). Spearman's linear correlation analysis was performed to estimate the relationship strength between endogenous shikimic acid concentration and the measured whole-plant rate response parameters.

The null hypothesis ( $H_0$ ) that the quantitative trait data represented a random sample from a normally-distributed population was tested by the univariate Shapiro–Wilk test and was accepted if the  $P$ -value for  $W_{100}$  was  $\geq 0.05$ ; otherwise, the variance ( $\sigma^2$ ) of the data was normalized by natural-log transformation (Shapiro and Wilk 1965). ANOVA was performed on individual quantitative traits considering taxon and family nested within taxon as fixed and random effects, respectively. Therefore, significant taxon effects were tested against the



$\sigma^2$  among families within taxon and family effects against the residual error. Since all quantitative traits were balanced, type I sums of squares were used to test the  $H_0$  of equal means among taxa. Fisher's  $\text{LSD}_{\alpha=0.05}$  test was performed for comparisons among taxa means when ANOVA identified significant taxon effects. When the normality assumption was not met, Kruskal–Wallis analyses (PROC NPAR1WAY) compared the  $H_0$  that the data were sampled from taxa with the same median. If accepted, *post hoc* non-parametric separation was conducted by Dunn's test (Bonferroni's method) (Conover 1999). Additionally, PROC VARCOMP was employed to partition the total phenotypic  $\sigma^2$  into the different ANOVA components for traits that converged Shapiro–Wilk's normality assumption;  $\sigma^2$  components were then estimated with the restricted maximum likelihood (REML) method and used to calculate the intraclass correlation coefficient:

$$t = \frac{\sigma_b^2}{\sigma_b^2 + \sigma_w^2} \quad [\text{Equation 1}]$$

Where  $\sigma_b^2$  and  $\sigma_w^2$  corresponded to the  $\sigma^2$  between and within taxon, respectively.

Phenotypic intermediacy of the  $F_1^H$ , as it related to the parents, was tested by a character count procedure using trait means based on an intermediate versus non-intermediate one-tailed sign test (Wilson 1992). The  $H_0$  that the residuals were multivariate normally distributed was tested by Mardia's kurtosis ( $\gamma_2$ ) analysis (Mardia 1970). PROC PRINCOMP was invoked to test whether the data covariance matrix was singular. If rejected, squared Mahalanobis distances ( $d_i^2$ ) from the centroid were computed for chi-square ( $\chi^2$ ) quantile–quantile (Q–Q) comparisons of multivariate normal data; 75% confidence intervals were constructed from standard deviation ( $\sigma$ ) estimates of  $g(z)$  as indicated by Chambers et al. (1983). Biometric indicators that converged Mardia's assumption were then subjected to canonical discriminant function (CDF) analysis utilizing PROC CANDISC (Thompson 1984). Taxon clustering of group averages used Mahalanobis' distance matrix based on the unweighted pair-group method with arithmetic mean (UPGMA; PROC CLUSTER); concomitantly, PROC TREE was used to display the phylogenetic relationship between taxon.

Homogeneity  $\chi^2$  tests according to Cochran–Mantel–Haenszel statistics compared the phenotypic segregation ratios of  $F_1^H$ ,  $F_2^H$ ,  $BC_c^H$ , and  $BC_r^H$  full-siblings for glyphosate resistance and if non-significant, the data among families were combined for further analysis. The observed phenotypic R, IR, and S segregation ratios were compared against the expected Mendelian proportions for the proposed genetic model and tested with a  $\chi^2$  goodness-of-fit (GOF) test.

## Results

### Parental populations differed in their response to glyphosate

#### *C. canadensis* is more resistant to glyphosate than *C. ramosissima*

LOF and coefficient of determination estimates for biomass ( $F_{\text{obs}} = 0.33$ ,  $P = 0.98$ ;  $R^2_{(\text{pseudo})} = 0.82$ ) and shikimic acid ( $F_{\text{obs}} = 1.36$ ,  $P = 0.15$ ;  $R^2_{(\text{pseudo})} = 0.92$ ) data confirmed appropriateness of the log-logistic model to describe plant development as a function of increasing glyphosate rates. *C. ramosissima* from Ames, IA was uniformly killed at 0.85 kg glyphosate  $\text{ha}^{-1}$ . Conversely, the Houston, DE *C. canadensis* population required at least 3-fold that rate to reduce biomass accumulation by 50% and an approximately 7-fold to inflict 50% mortality in the population (Table 1). Glyphosate toxicity symptoms included plant stunting, leaf chlorosis, and necrosis that developed from the meristems and leaf tips to the rest of the plant. At the 2.0 kg glyphosate  $\text{ha}^{-1}$  rate, *C. canadensis* plants developed marginal injuries consistent with glyphosate toxicity, demonstrated growth rates analogous to non-treated plants, and reached reproductive stage. Comparing *C. ramosissima* and *C. canadensis*  $GR_{50}$ 's estimated a  $|\lambda_{50}|$  value of 1.60 ( $F_{\text{obs}} = 1.77$ ;  $P < 0.01$ ), affirmation of a different response to glyphosate between the populations. These results parallel reports of another *C. canadensis* population from Tennessee (Mueller et al. 2003).

#### *Shikimic acid accumulates in glyphosate susceptible plants*

In plants, glyphosate causes cytoplasmic accumulation of the substrate and unphosphorylated substrate of EPSPS at a 1:20 proportion of 3-phosphoshikimate (3PS):shikimic acid (Gout et al. 1992). Ultimately, putative phosphorylases hydrolyzed the

phosphoryl group in 3PS and the aromatic compound is accumulated as shikimic acid in vacuoles (Holländer-Czytko and Amrhein 1983). Monitoring endogenous shikimic acid is therefore an indirect estimate of the level of EPSPS inhibition by glyphosate and serves as a pragmatic method to corroborate whole-plant efficacy to the herbicide (Harring et al. 1998). Shikimic acid determinations suggested that twice the rate of glyphosate was required to inhibit 50% of EPSPS in the *C. canadensis* resistant population compared to the susceptible *C. ramosissima* (Table 1). Untreated *Conyza* plants contained 7 to 14  $\mu\text{mol}$  shikimic acid  $\text{g}^{-1}$  dry tissue, which increased sigmoidally to a maximum of 150  $\mu\text{mol}$  with increasing glyphosate rates (Figure 1). Concurrently, endogenous shikimic acid negatively correlated with biomass ( $r^2 = -0.64$ ;  $P < 0.001$ ) and positively with visual injury ( $r^2 = 0.76$ ;  $P < 0.001$ ), thus supporting whole-plant rate response results. Levels of 0.5  $\mu\text{mol}$  shikimic acid  $\text{g}^{-1}$  fresh tissue in untreated plants and  $> 5.7$   $\mu\text{mol}$  in a glyphosate resistant *C. canadensis* biotypes treated with 3.8 kg glyphosate  $\text{ha}^{-1}$  were previously reported (Mueller et al. 2003).

### Hybridization between *C. canadensis* and *C. ramosissima*

#### *Taxa are compatible yet demonstrate low hybridization levels*

Pistillate florets in emasculated *C. ramosissima* capitula contained  $< 1$  % viable achenes in the absence of pollen (Table 2). Fertilization of pistillate florets under these conditions probably originated from pollen of perfect florets prior to anthesis or pollen of perfect florets incompletely excised during emasculation. Under normal environments approximately 60 % of total *C. ramosissima* florets are fertilized (Figure 2). Therefore, we estimated that emasculation of *C. ramosissima* was 98 % effective. Assisted hybridization ranged from 0 % to near 7 % in the *C. canadensis* to *C. ramosissima* crosses and 0% to near 9% in the reciprocal crosses, although five in ten *C. ramosissima* and two in ten *C. canadensis* families contained no identifiable hybrid progeny (Table 2). In contrast, high introgression levels ( $> 95\%$ ) of the *R*-allele were observed in the artificial-unidirectional *C. canadensis* to *C. ramosissima* crosses. The observed levels of glyphosate S individuals ( $< 2$  %) in artificial crosses mirrored those of emasculation inefficiency estimates (2%), suggesting that some self-fertilization occurred prior to anthesis. Glyphosate toxicity symptoms in S hybrids were consistent with those observed in the *C. ramosissima* parent.

Collectively, these data suggested that while the levels of assisted hybridization between *C. canadensis* and *C. ramosissima* were relatively low (< 4.1 %), the taxa are genetically compatible and thus the potential for interspecific hybridization is significant.

*The  $F_1^H$  shares an intermediate phenotype between both parents*

**Differences between taxa account for most of the phenotypic variance ( $\sigma^2$ ).**

Differences among families within taxa were significant for approximately half of the quantitative traits evaluated (Table 3). Similarly, differences among families for the ANOVA conducted on individual taxon were significant for 17 traits in at least one taxa. Maternal family effects were significant in 9 and 10 quantitative traits within *C. ramosissima* and the  $F_1^H$  populations, respectively, and in approximately half of the *C. canadensis* traits (Table 3). Only the characters rosette leaf length, cauline leaf trichomes, and achene length had significant maternal family effects across taxon. Intraclass correlation coefficients were generally (85%) different from zero and most (72%) were greater than 0.90 (Table 3). Combined with the ANOVA, data therefore suggested that most of the  $\sigma^2$  was accounted for by differences between the *Conyza* taxa. Furthermore, we assumed that this  $\sigma^2$  was attributable primarily to genetic differences among taxa rather than to interactions with the environment as populations developed under controlled greenhouse conditions.

**Morphometric data suggests that the  $F_1^H$  is intermediate to both parents.**

Significant differences among the studied taxa were attained for all 27 quantitative traits evaluated (Table 3). *C. canadensis* produced wider rosettes with fewer leaves than *C. ramosissima* (Table 4). Furthermore, both *C. canadensis* leaf dimorphisms were longer, wider, and possessed more dentations than *C. ramosissima*. In contrast, *C. ramosissima* was profusely branched, had narrower stems, and developed more pubescent leaves with greater density (SLA) than *C. canadensis* (Table 4). Capitula dimensions and the number of individual and total florets were greater in *C. canadensis*, while *C. ramosissima* produced longer achenes. Morphometric traits were in agreement with previous taxonomical descriptions and permitted appropriate segregation of the taxon, even during early plant developmental stages (Gleason and Cronquist 1991).

Five of the 27 morphometric traits evaluated did not meet the character count assumption that the  $F_1^H$  was intermediate to both parents (Table 4;  $D = 22$ ). Since conditions for the DeMoivre–Laplace theorem were met ( $np$  and  $nq \geq 5$ ), normal approximation was used for probability estimation rather than a binomial distribution. Rosette leaf number, cauline branch number, cauline SLA, and capitula length measurements in the  $F_1^H$  tended to supersede those of either parent, however only the trait capitulum shape was significantly different (Table 4). The calculated one-tailed sign test value for phenotypic intermediacy was significant ( $z_+ = 3.27$ ;  $P = 0.001$ ) and hence prompted rejection of the  $H_0$  for equal sign proportions of morphometric traits. The collective phenotypic data therefore supported the principle that the  $F_1^H$  represented an intermediate between both parents.

**The  $F_1^H$  while intermediate, is more similar to the *C. ramosissima* parent.**

Multivariate normality analysis elucidated that only 17 of the 27 morphometric traits converged to Mardia's statistics ( $\gamma_2 = 328.2$ ,  $P = 0.08$ ) and thus were combined for multivariate discriminant analysis; the pistillate florets trait was eliminated from the analysis as inclusion instigated a singular covariance matrix. The  $H_0$  that data arose from populations with a common distribution was tested by Q–Q plots; linearity of data points along the expected mean vectors and allocation within the estimated 75% confidence intervals suggested non-departure from normality (Figure 2). Most (81%) of intraclass correlation coefficients in multivariate normally distributed traits possessed values greater than 0.70, hence suggesting negligible interference of correlations among families that could potentially distort the interpretation of the CDF analysis.

Univariate statistics for differences among taxa means were significant ( $P < 0.0001$ ) for all traits (Table 5), as were Wilks' exact multivariate statistics for comparison among taxa ( $\lambda = 0.004$ ;  $F = 229.9$ ;  $P > 0.0001$ ). CDF analysis estimated that the first (Can1) and second (Can2) canonical variates accounted for 97 % and 82 % of the total quantitative trait  $\sigma^2$ , respectively. Both cauline leaf trichomes (Can1 = 1.61; Can2 = -2.40) and total florets (Can1 = -2.07; Can2 = -1.08) traits contributed more heavily to the canonical variates than the rest of morphometric traits and contained the highest  $R^2$  values (Table 5). Individuals with high positive Can1 estimates had more cauline leaf trichomes than plants with high negative Can2

values; conversely, plants with high negative Can1 and Can2 values had below average total florets per capitulum (data not shown). Concomitantly, taxa clustered in discrete sections within the canonical graph, *C. ramosissima* (6.8) and the  $F_1^H$  (1.3) were positive along the Can1 axis, while *C. canadensis* was diametrically negative (-8.2). Fewer taxa divergence was obtained along the Can2 axis (Figure 2). Since Can1 had the greatest discriminatory power, morphometric traits with total-sample standardized canonical coefficients (SCC)  $> \pm 0.60$  allowed for appropriate separation between taxa (Table 5). *C. ramosissima* had a compact stature with narrower rosettes (SCC = -0.85), more pubescent (SCC = 1.61), denser (SCC = 0.63), but shorter (SCC = -0.64), and slender (SCC = -0.62) leaves, and fewer florets per capitulum (SCC = -2.07) than *C. canadensis*; the  $F_1^H$  was intermediate to these parameters. Taxa separation was also discernable by group averages based on the UPGMA. Divergence in Mahalanobis distances was greatest among parents ( $d_t^2 = 226.5$ ;  $F = 630.2$ ;  $P < 0.0001$ ) and approximately half and one fourth that square distance was estimated for comparisons between the  $F_1^H$  and *C. canadensis* ( $d_t^2 = 107.5$ ;  $F = 299.1$ ;  $P < 0.0001$ ) and *C. ramosissima* ( $d_t^2 = 53.7$ ;  $F = 149.4$ ;  $P < 0.0001$ ), respectively. Hierarchical clustering formed a dendrogram with a common root node for the taxon (Figure 2). The *C. ramosissima* and  $F_1^H$  leaves clustered within a single branch, whereas *C. canadensis* clustered to the common parent node. While the character count procedure lend credence to the thesis of an intermediate hybrid phenotype, CDF analysis and UPGMA strongly emphasized that the  $F_1^H$  was more similar to *C. ramosissima* than the *C. canadensis* parent.

#### *Postzygotic barriers do not limit reproduction of the $F_1^H$*

Partial correlation estimates ( $r^2 = 0.76$ ;  $P = 0.001$ ) associated with the sums of squares and crossproducts (SSCP) matrix suggested a strong relationship strength between the viability experiments repeated in time. Univariate ( $F = 0.14$ ;  $P = 0.71$ ) and multivariate (Wilks'  $\lambda = 0.99$ ;  $P = 0.71$ ) tests for between time effects were not significant, therefore no difference in repeated measures was inferred and data were combined for further analysis. Previous research reported near perfect *C. canadensis* germination under light and constant

28 C conditions (Shontz and Oosting 1970). Viability of *C. canadensis* achenes under our conditions ranged from 17 % to 71 % within families with a mean of 46 %, which was not significantly different ( $\text{LSD}_{\alpha 0.05} = 12 \%$ ) from the 57 % mean viability of *C. ramosissima* achenes (Figure 2).

Evidence for hybrid inviability was apparent in the significant 28% depression of viability and 23% increase in non-viable  $F_1^H$  achenes. Artificial crosses estimated hybridization levels of 95% to 100% in the fertile hybrid zygotes within families (Table 2). Viability tests nonetheless confirmed that approximately half of the available ova in *C. ramosissima* were not fertilized by *C. canadensis* spermatozoa, an argument that some level of genetic incompatibility existed among parents (Figure 2). While physical damage of *C. ramosissima* capitula during emasculation could have contributed to the inviability of hybrid zygotes, epistatic, homeotic transformations, or interactions of complementary genes may have also prompted abortion of embryos in  $F_1^H$  achenes. The presence of a hybrid sterility reproductive barrier was disregarded as self-pollinated  $F_1^H$  plants ( $F_2^H$ ) produced embryos with approximately twice the level of viability of their progenitors. Estimates of  $F_2^H$  viability were significantly less than for either parent, an indication that some level of self-infertility remained within hybrids and evidence that the inviable  $F_1^H$  zygotes probably arose from genic incompatibilities rather than the physical stress of emasculation (Figure 2).

Backcrosses of  $F_1^H$  to *C. canadensis* and *C. ramosissima* produced a viable progeny, 21% and 25 % respectively, confirming successful gene flow among the evaluated *Conyza* taxa through the hybrid intermediate. Establishment of a hybrid taxa in the environment is often limited by postzygotic reproductive barriers (Barton 2001). While the germination of  $F_1^H$  achenes was less than the parents, the collective viability data suggested that the *Conyza* hybrid taxa was fertile and probably capable of natural establishment in the environment.

#### *Transgressive segregation: a common trait of the $F_2^H$*

Hybrid progenies demonstrated marked traits of transgression (Rieseberg et al. 1999). Most  $F_2^H$  phenotypes manifested similarities to either the specific epithets *canadensis* or

*ramosissima*; however, extreme transgressive traits were also observed ranging from glabrous to pubescent leaves and stems, oblanceolate to subulate leaves with dentated to strait margins, green to purplish midribs, irregular arrangements of lateral leaf nervures, and profusely branched single-stems. Generally capitula morphology was a preserved trait among  $F_2^H$  individuals. Transgressive segregation was also discernable in whole-plant response to increasing glyphosate rates. Compared to the biomass accumulated by either *C. canadensis* ( $\sigma^2 = 0.67$ ), *C. ramosissima* ( $\sigma^2 = 0.42$ ), or the  $F_1^H$  ( $\sigma^2 = 0.87$ ),  $F_2^H$  rosettes demonstrated greater variance ( $\sigma^2 = 1.17$ ) in response to glyphosate. Similarly, greater variation in the level of endogenous shikimic acid accumulation and visual injury was observed in the  $F_2^H$  (data not shown). The  $F_2^H$  was also characterized by the unexpected death of plants (< 5%) during early rosette stages. We ascribed these low lethal frequencies to possible deleterious homeotic transformations, epistatic or complementary genes interactions in  $F_2^H$  plants.

## Genetics of glyphosate resistance

### *Parents represent near-homozygous lineages*

Anecdotal accounts by the City of Ames suggested that the *C. ramosissima* population evolved in the absence of glyphosate. Application of 0.40 kg glyphosate ha<sup>-1</sup> inflicted visual injury levels of 30% to 60% and retarded plant development compared to untreated *C. ramosissima* plants, although treated plants reached reproductive stage by recovering from these injuries within 2 to 4 weeks. The 0.40 kg ha<sup>-1</sup> rate therefore allowed for non-destructive identification of the S phenotype. Rates of 0.85 kg glyphosate ha<sup>-1</sup> resulted in uniform control of *C. ramosissima*, suggesting that the population was near-homozygous S to glyphosate. The original *C. canadensis* and subsequent populations obtained through two cycles of recurrent selection demonstrated no mortality at the glyphosate rate lethal to *C. ramosissima*; however, the populations developed 0 % to 20 % visual injuries when treated with 2.0 kg glyphosate ha<sup>-1</sup>. Comparison of the estimated  $GR_{50}$ 's for these *C. canadensis* populations provided non-significant  $|\lambda_{50}|$  values (data not shown), demonstrating that the populations possessed a similar response to glyphosate. Therefore, we



assumed that the *C. canadensis* populations represented a near-homozygous R lineage to glyphosate.

*Glyphosate resistance is overdominant in the  $F_1^H$*

Maternal inheritance of glyphosate resistance was investigated in the 7 and 12  $F_1^H$  plants originated from the assisted reciprocal crosses (Table 2). Treatment with glyphosate at  $2.0 \text{ kg ha}^{-1}$  confirmed that  $F_1^H$  rosettes, irrespective of the maternal origin, possessed a similar resistance level to glyphosate (data not shown). Therefore, we rejected the supposition that the R-allele resided in the cytoplasm of *C. canadensis* cells. In all reports to date, herbicide resistance is overwhelmingly governed by a nuclear-monogenic event (Gasquez 1997).

The unidirectional *C. canadensis* to *C. ramosissima* artificial cross produced  $F_1^H$  plants with quantitative traits predominantly intermediate to both parents (Figure 5). The  $F_1^H$  produced larger rosettes than the *C. ramosissima* parent and more and denser (SLA) leaves than *C. canadensis*. This divergence in morphometric parameters resulted in an apparent heterotic response recorded at the 10 to 12 cm diameter rosettes stage (Figure 1). Compared to either parent, higher glyphosate rates were required to reduce biomass accumulation or cause mortality in the  $F_1^H$  population, whereas narrower differences were observed in the pattern of endogenous shikimic acid accumulation (Figure 1; Table 1). The divergence in glyphosate response was confirmed by  $|\lambda_{50}|$  value estimations for comparisons of the  $F_1^H$  and *C. canadensis* ( $F_{\text{obs}} = 1.74$ ;  $P < 0.01$ ) or  $|\lambda_{50}|$  value estimations with *C. ramosissima* ( $F_{\text{obs}} = 1.76$ ;  $P < 0.01$ ). Not only did  $F_1^H$  rosettes demonstrate a vigorous growth that probably required higher glyphosate rates to inhibit growth, but leaves produced more trichomes than the R parent which could hinder glyphosate absorption into the plant (Table 4). The  $F_1^H$  response to glyphosate failed to obey the additive, dominant, or hybrid susceptibility models that describe plant resistance in hybrid populations; rather, the behavior was best explained by the hybrid resistance hypothesis, which predicts greater resistance in hybrids compared to the independent performances of the progenitors (Fritz et al. 1994).

*The R-allele segregates following a 1:2:1 model*

The supposition that glyphosate resistance in *Conyza* was governed by a single allele inherited in an incompletely-dominant model was tested by ascertaining the  $F_2^H$ ,  $BC_c^H$ , and  $BC_r^H$  segregation ratios. Efficacy evaluated two weeks post application of 2.0 kg glyphosate  $ha^{-1}$ , a rate that differentiated R and S phenotypes in the parental populations, discerned three distinct segregates in  $F_2^H$  families – R, IR, and S phenotypes. Exact GOF tests based on the  $H_0$  that the observed segregation ratios followed a 1:2:1 Mendelian distribution provided non-significant  $\chi^2$  values for all  $F_2^H$  families, substantiating the appropriateness of the partially-dominant monogenic model (Table 6). Concomitantly, homogeneity analysis ( $\chi^2 = 5.72$ ;  $P = 0.77$ ) confirmed that the combined  $F_2^H$  data converged to the 1:2:1 genetic model. Backcross of  $F_1^H$  to *C. canadensis* ( $BC_c^H$ ) and subsequent treatment of progeny with 2.0 kg glyphosate  $ha^{-1}$  discerned only R and IR phenotypes. The observed segregation ratios among  $BC_c^H$  families were homogenous ( $\chi^2 = 4.40$ ;  $P = 0.88$ ) and the collective data were consistent with the expected 1:1 ratio (Table 6). Similarly,  $F_1^H$  backcross to *C. ramosissima* ( $BC_r^H$ ) resulted in families with homogenous IR and S segregation ratios ( $\chi^2 = 5.81$ ;  $P = 0.76$ ) that obeyed the partially-dominant model. The moderate increases of R and S phenotypes in backcross families were attributable to emasculation imperfections or self-fertilization prior to anthesis (Table 2). Regardless, the combined backcross data corroborated our previous assertion that the *C. canadensis* and *C. ramosissima* parental populations comprised near-homozygous lineages.

## Discussion

### *Conyza* hybridizes in nature

Phylogenetic boundaries in *Conyza* are not clearly demarcated and considerable phenotypic variation in the taxa has been reported with extreme phenotypes occurring in response to adverse environmental stimuli (Nesom 1990). Without *a priori* knowledge, we embarked on a project to assess potential hybridization of *Conyza* species and better understand the phylogenetic relationship between *C. canadensis* and *C. ramosissima*, two

weedy plants in US agroecosystems. Our work herein suggests that the studied taxa are genetically compatible, capable of introgression, and that interspecific progenies are vigorous and fertile. We speculate that the relatively high genetic compatibility among the taxa is associated with the common diploidy structure ( $2n = 18$ ) that allows for successful chromosome pairing during meiosis. Both *C. canadensis* and *C. ramosissima* represent sibling species as elucidated by nrDNA internal transcribed spacers (ITS) analysis, which clustered taxa in a single branch within group VI of the *Erigeron* and allied Asteraceae cladogram (Noyes 2000). This phylogenetic analysis estimated a recent speciation event between the *ramosissima* and *canadensis* epithets and provided further support to our thesis of genetic compatibility between the taxa.

The likelihood of native hybridization in *Conyza* is probably low given the autogamous reproduction in the genus. Entomophily was reported in *C. canadensis* (Weaver 2001), although the contribution to allogamy or interspecific gene transfer remains unknown. Prior documentation of hybridization between *C. canadensis* and *C. ramosissima* is, to our knowledge, nonexistent. However, since the early descriptions, plants with common traits between both taxa have been cited (Michaux 1803). Voss (1996) reported depauperate *C. canadensis* biotypes in the upper Michigan peninsula with glabrous to pubescent patterns and ramification near or below the main stem, characteristics that we observed in the transgressive  $F_2^H$ . In Canada, Darbyshire (1990) described profusely branched *C. canadensis* biotypes that resembled our  $F_1^H$  but he attributed those abnormalities to a loss of apical dominance. Since environmental factors can moderate the phenotypic expression of *C. canadensis* and *C. ramosissima*, some contend that the taxa are conspecific and argue that the latter represents depauperate extremes of the highly-plastic *canadensis* epithet (Darbyshire, S. Personal Communication). Because *C. canadensis* and *C. ramosissima* grown in the greenhouse preserved their distinctive phenotype, we refuted the argument of a conspecific taxon. We offer the alternative that perhaps some of these extreme phenotypes represent transgressive hybrids misinterpreted as either progenitor. Understanding interspecific gene transfer and the interactions of genetic and environmental stimuli affecting

the phenotypic expression of *Conyza* are factors that deserve further investigation (Voss 1996).

Documentation of hybridization in Conyzinae is at present restricted to Europe. In the British Isles, hybridization between *C. canadensis* and blue fleabane (*Erigeron acer* L.) was reported to engender weak and apparently sterile plants with few capitula; the nothotaxa was classified as *Conyzigeron x huelsenii* (Vatke) Rauschert (Stace 1975). Hybrids of unknown fecundity, namely *C. x flahaultiana* (Thell.) Sennen and *C. x daveauiana* Sennen in Spain and France, originated from crosses between *C. canadensis* and hairy fleabane (*C. bonariensis* (L.) Cronq.) and between *C. bonariensis* and Sumatran fleabane [*C. sumatrensis* (Retz.) E. Walker], respectively (McClintock and Marshall 1988). In the Iberian peninsula, *C. x rouyana* Sennen arose from the hybridization between broadleaf fleabane (*C. albida* Willd. ex Spreng.) and *C. canadensis* (Carretero, J.L. Personal Communication). Additionally, Thébaud and Abbott (1995) stated that in France, *C. sumatrensis* and Spanish fleabane (*Conyza blakei* Cabr.) cross under natural environments and produce a hybrid with moderate (30 %) fertility. In Belgium, Verloove and Boulet (2001) reported that some xenophyte *Conyza* populations identified as *C. floribunda* were in fact *C. canadensis* x *C. sumatrensis* hybrids. Furthermore, *Conyza* plants with an intermediate phenotype between *C. canadensis* and *C. bonariensis* were discovered near a demolition area in Antwerp, Belgium and thereafter confirmed in France, Great Britain, Portugal; the nothotaxa was classified as *C. x mixta* Fouc. & Neyr. (Verloove, F. Personal Communication). More recently, Šída (2003) reported a putative hybrid between *C. bonariensis* and trilobus fleabane (*Conyza triloba* Decne.) in the Czech Republic.

Loss of vigor is apparently a common motif within the hybrid European *Conyza*. We speculate that ploidy may be a significant barrier determining the success of hybridization in Conyzinae; more compatible and vigorous hybrids would be expected from crosses between the allopolyploids ( $2n = 54$ ) *C. sumatrensis*, *C. floribunda*, and *C. bonariensis* than with the diploid ( $2n = 18$ ) *C. canadensis*. Just as geography delimits the major groups in Asteraceae (Nesom 1994), spatial isolation is probably the principal species barrier within Asteraceae. Disturbances stimulate interactions between phylogenetically related taxa separated by

geography (allopatry) and thus allow for the exchange of genetic material between unstructured random-mating (panmictic) Asteraceae populations.

### **The hybrid formed probably without an increase in ploidy**

The role of hybridization as a mechanism for plant speciation was first proposed by Linnaeus (Linné 1760); his hypothesis however ignored the significant contributions of segregation and sterility in determining hybrid speciation. Further experimentation by Winge (1917) and Muntzing (1932) demonstrated that fertile and stable hybrids could arise by either chromosome number doubling (allopolyploidy) or recombinational speciation without polyploidization (homoploidy). In the absence of karyological studies, the ploidy of *C. canadensis* x *C. ramosissima* remains unknown. Nonetheless, we contend that the segregation and compatibility data alludes to the formation of an interspecific hybrid without an increase in ploidy. Man-made allopolyploids often display homeotic transformations and aberrant chromosomal rearrangements that result in gene silencing, hybrid instability, and lethality (Comai 2000). These phenomena were apparently absent from the *Conyza* hybrid, as the interspecific hybrids demonstrated a vigorous growth and marginal ( $< 5\%$ )  $F_2^H$  lethality (Figure 1; Table 4). Additionally, homeologous recombination in neo-allopolyploids typically destabilizes genomes by chromosomal deletions or expansions, and homeologous pairing often hinders meiosis through the formation of uni- or tri-valents (Comai 2000). The  $F_1^H$  however demonstrated negligible postzygotic reproductive barriers was genetically compatible with the parental taxa (Figure 2). Finally, allopolyploids often preserve homeologous integrity by hindering intergenomic recombination, whereas in homoploids, numerous chromosome combinations form through chiasmata thus allowing recombination of the parental genomes (Comai 2000). Since recurrent selection suggested that the *C. canadensis* and *C. ramosissima* populations were near-homozygous and the fact that  $F_2^H$  families segregated for glyphosate resistance implied that the *C. canadensis* and *C. ramosissima* genomes coalesced. This characteristic of segregation is typically associated with homeologous recombination in homoploids.

### Speciation may depend on hybrid fitness and niche divergence

The low hybridization (< 12%) in non-emasculated crosses and genetic compatibility estimated between the evaluated taxa suggested that *Conyza* population in nature probably evolve as contiguous populations in a common geographic range (parapatry) (Table 2). Under natural environments hybridization may occur at lower or higher frequencies than these estimates depending on entomophilous or anemophilous interactions and other environmental effects such as competition and resource availability which modulate flowering time in *Conyza* (Thébaud et al. 1996). Regardless, the fact that the *C. canadensis* x *C. ramosissima* hybrid was stable and fertile connotes that fitness and habitat divergence are essential factors determining the adaptation of *Conyza* hybrids in the environment.

Models for homoploid speciation suggest that superior hybrid competitiveness results in the rapid displacement of the parental taxa, while under a fitness disadvantage, either the hybrid taxa becomes extinct or it coexists with the parents, provided that adequate niche differentiation exists (Rieseberg 1997). Hybrid neospecies are rarely better fit than their well-adapted congeners. Low initial frequencies, reduced fertility and viability, and competitive disadvantage with respect to parents often lead to hybrid extinction (Wolf et al. 2001). We estimated adequate hybrid fitness as the evaluation of the  $F_1^H$  showed heterosis and overdominance for glyphosate resistance compared to the parental taxa (Table 1; Figure 1). Furthermore, the  $F_1^H$  developed capitula throughout the multiple and profusely branched stems and thus produced an equal number, if not more, achenes than either parent. This reproductive behavior could compensate for the estimated depression in  $F_1^H$  achene viability (Figure 2).

Ascertaining hybrid fitness and the subsequent ecological displacement of *Conyza* taxa in different areas (vicariation) would require additional experimentation. Nevertheless, we believe that several hybrid traits would serve as ecological advantages, at least under current agroecosystems.  $F_1^H$  rosettes may be competitive by generating more and denser leaves than *C. canadensis* and producing wider rosettes than *C. ramosissima* (Table 4) thus accumulating greater biomass than either parent (Figure 1). In addition,  $F_1^H$  possessed leaves with higher trichome densities than the glyphosate resistant parent, a trait that may reduce

uptake and hinder herbicide efficacy. Furthermore,  $F_1^H$  plants produced multiple branches which may facilitate recovery from herbicide applications through meristematic regrowth (Table 4). Achene adaptations for dispersion by wind (anemochory), the capacity to overwinter (therophyte), and prolific achene production make *C. canadensis* extremely competitive under no tillage agroecosystems. However, the species is poorly adapted to production systems where tillage is used as a management strategy (Brown and Whitwell 1988). Hence, the larger  $F_1^H$  achenes may provide an ecological advantage over *C. canadensis* by allowing emergence from deeper soil profiles in tilled agroecosystems (Table 4). According to Barton (2001), reasons for higher hybrid fitness include the (1) occurrence of diverse phenotypes through transgression that may have ecological advantages under particular environments, (2) reconstruction of an 'ancestral linkage' which was competitive in the past and is probably adept to present environments, and (3) coalescence of previously disjunct gene sets that may have a positive impact on fitness. Hybridization may therefore explain some cases of niche differentiation and provide the crude materials for the rapid adaptation of novel hybrids in the environment (Rieseberg et al. 1999).

#### **Hybridization may complicate herbicide resistance management**

The impact of hybridization on glyphosate resistance management would depend on the native introgression levels of the *R*-allele and the stability, fertility, and fitness of the hybrid with respect to parents. Herein we demonstrated that the *C. canadensis* x *C. ramosissima* nothotaxa are stable, fertile, and competitive (Figure 1; Figure 2). In addition, successful *R*-allele introgression occurred from *C. canadensis* to *C. ramosissima* through the  $F_1^H$  (Table 6). Furthermore, the  $F_2^H$  transgressed for many traits which may facilitate the adaptation to different agroecological niches. These circumstances would certainly complicate management of glyphosate resistance and the containment of resistance genes within agroecosystems. Absent from this hypothesis, however, are the natural hybridization frequencies in *Conyza*. Native hybridization frequencies in the European *Conyza* approximate 50 plants in thousands of individuals, although 60% of these hybrids were infertile (Thébaud and Abbott 1995). We would expect higher hybridization frequencies

than those reported in Europe since the parents are compatible and the hybrid demonstrated negligible postzygotic reproduce barriers. Even low initial hybrid frequencies within the population could increase in time, as *Conyza* can produce more than 240,000 achenes per growing season and disperse achenes up to 30 m in 16 km h<sup>-1</sup> wind (Muenscher 1935; Dauer et al. 2003). Interspecific hybridization may therefore affect the extinction rates in the environment and serve as a mechanisms for the dissemination of transgenic and herbicide resistance genes (Chèvre et al. 2000; Franssen et al. 2001). Farmers should consider the potential for hybridization when developing programs aimed at managing herbicide resistance weeds. Production systems that depend on a single herbicidal chemistry for weed control should be combined with alternative management tactics, thus mitigating the evolution of herbicide resistance and maintaining the sustainability of current agroecosystems.

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**Table 1.** Comparison of glyphosate resistance in the parental *Conyza canadensis* and *C. ramosissima* populations and the interspecific hybrid ( $F_1^H$ ) and hybrid progeny ( $F_2^H$ ).

Numbers in parenthesis designate the 95% lower and upper confidence intervals ( $GR_{50}$ ;  $I_{50}$ ) or fiducial limits ( $LD_{50}$ ) of the estimated parameter.

Taxon	$GR_{50}^a$	$LD_{50}^b$	$I_{50}^c$
<i>C. ramosissima</i>	0.69 (0.38–0.99)	0.68 (0.35–1.04)	1.84 (1.59–2.10)
<i>C. canadensis</i>	2.29 (1.51–3.07)	4.79 (3.29–7.02)	3.80 (3.44–4.16)
$F_1^H$	3.58 (2.42–4.74)	6.23 (4.21–9.20)	4.08 (3.63–4.53)
$F_2^H$	3.93 (2.27–5.59)	3.16 (1.85–5.68)	3.27 (2.93–3.61)

<sup>a</sup> = glyphosate rate in kg ha<sup>-1</sup> that reduced biomass accumulation by 50%.

<sup>b</sup> = glyphosate rate in kg ha<sup>-1</sup> that inflicted 50% mortality in the population.

<sup>c</sup> = glyphosate rate in kg ha<sup>-1</sup> that increased endogenous shikimic acid accumulation by 50%.

**Table 2.** Efficiency of emasculation (EE) in *Conyza ramosissima* capitula and frequencies of assisted and artificial hybrid formation between *C. canadensis* and *C. ramosissima*. Uncrossed values correspond to the germination (G) of estimated achenes (EA) per family. For crosses, percent hybridization (PH) was estimated from the frequency of glyphosate resistant (R), susceptible (S), and plants with a hybrid phenotype (H) within the family.

Family	Uncrossed <sup>a</sup>			Assisted cross <sup>b</sup>						Artificial cross <sup>c</sup>		
	<i>C. ramosissima</i>			<i>C. ramosissima</i>			<i>C. canadensis</i>			<i>C. ramosissima</i>		
	EA	G	EE <sup>d</sup>	S	H <sup>e</sup>	PH <sup>f</sup>	R	H	PH	S	H	PH
1	120	1	99.2	22	0	0.0	34	2	5.6	0	29	100.0
2	110	0	100.0	26	1	3.7	21	2	8.7	1	22	95.7
3	80	0	100.0	27	2	6.9	27	1	3.6	0	25	100.0
4	130	1	99.2	23	0	0.0	26	0	0.0	1	27	96.4
5	90	1	98.9	34	1	2.9	25	2	7.4	1	22	95.7
6	100	0	100.0	21	0	0.0	30	0	0.0	0	29	100.0
7	90	0	100.0	24	0	0.0	25	1	3.8	0	29	100.0
8	130	0	100.0	29	2	6.5	33	1	2.9	1	24	96.0
9	100	1	99.0	26	0	0.0	29	1	3.3	1	30	96.8
10	80	0	100.0	25	1	3.8	30	2	6.2	0	30	100.0
Total	1030	4	99.6	257	7	2.6	280	12	4.1	5	267	98.2

<sup>a</sup> = *C. ramosissima* capitula emasculated pre-anthesis were covered with a DelNet<sup>®</sup> and allowed to mature in the absence of pollen.

<sup>b</sup> = *C. canadensis* and *C. ramosissima* inflorescences were covered with a DelNet<sup>®</sup> bag at anthesis and permitted to interact physically.

<sup>c</sup> = *C. ramosissima* capitula emasculated pre-anthesis were fertilized with *C. canadensis* pollen.

<sup>d</sup> = percent emasculation efficiency (EE) reported the proportion of non-viable achenes within the total evaluated.

<sup>e</sup> = plants demonstrated a hybrid phenotype (H) and resistance levels comparable to R individuals.

<sup>f</sup> = percent hybridization (PH) estimated the proportion of H plants within the total evaluated.



**Table 3.** Partitioning of the total variance ( $\sigma^2$ ) into taxon and family effects for the evaluated quantitative traits. Numbers represent  $F$ -values and probability (in parenthesis) estimates for the analysis of variance (ANOVA). The total  $\sigma^2$  between and within the *Conyza* taxon was utilized to calculate the intraclass correlation coefficients for each trait; underlined coefficients correspond to traits with a significant family effect ( $P < 0.05$ ) in ANOVA for individual taxa.

Stage <sup>a</sup>	Quantitative trait	Sources of variance <sup>b</sup>		Intraclass correlation coefficient <sup>c</sup>		
		between taxon	family within taxa	<i>C. canadensis</i>	Hybrid	<i>C. ramosissima</i>
Rosette	diameter (mm)	85.26 (< 0.0001)	2.33 (0.0151)	<u>0.98</u>	1.00	<u>0.99</u>
	leaf number	365.34 (< 0.0001)	0.4 (0.9364)	0.00	0.00	0.00
	leaf length (mm)	40.15 (< 0.0001)	2.45 (0.0109)	<u>1.00</u>	<u>0.95</u>	<u>1.00</u>
	leaf width (mm)	142.91 (< 0.0001)	2.34 (0.015)	<u>1.00</u>	1.00	1.00
	leaf shape	30.64 (< 0.0001)	3.8 (0.0002)	<u>1.00</u>	<u>1.00</u>	1.00
	leaf dentation <sup>d</sup>	314.24 (< 0.0001)	1.35 (0.2128)	NP <sup>e</sup>	NP	NP
	leaf trichomes (cm <sup>2</sup> )	56.14 (< 0.0001)	3.5 (0.0004)	<u>0.93</u>	1.00	<u>0.97</u>
	leaf area (mm <sup>2</sup> )	106.13 (< 0.0001)	1.56 (0.1282)	1.00	0.00	1.00
	leaf weight (mg)	132.37 (< 0.0001)	1.75 (0.077)	1.00	0.00	1.00
	SLA <sup>f</sup> (mm <sup>2</sup> mg <sup>-1</sup> )	302.88 (< 0.0001)	1.85 (0.0591)	<u>0.96</u>	<u>1.00</u>	0.87
Cauline	stem diameter (mm)	508.08 (< 0.0001)	2.01 (0.0386)	1.00	<u>1.00</u>	<u>0.91</u>
	branch number <sup>d</sup>	769.94 (< 0.0001)	1.21 (0.291)	NP	NP	NP

	leaf length (mm)	197.69 (< 0.0001)	3.79 (0.0002)	1.00	<u>1.00</u>	<u>0.94</u>
	leaf width (mm)	231.86 (< 0.0001)	2.09 (0.0306)	0.65	1.00	1.00
	leaf shape	13.19 (< 0.0003)	2.7 (0.0051)	<u>1.00</u>	<u>1.00</u>	0.00
	leaf dentation <sup>d</sup>	367.94 (< 0.0001)	0.65 (0.7537)	NP	NP	NP
	leaf trichomes (cm <sup>2</sup> )	294.88 (< 0.0001)	8.13 (< 0.0001)	<u>1.00</u>	<u>0.92</u>	<u>1.00</u>
	leaf area (mm <sup>2</sup> )	189.96 (< 0.0001)	2.92 (0.0026)	0.72	<u>1.00</u>	<u>0.86</u>
	leaf weight (mg)	202.89 (< 0.0001)	1.82 (0.0645)	0.29	<u>1.00</u>	<u>0.79</u>
	SLA (mm <sup>2</sup> mg <sup>-1</sup> )	19.85 (< 0.0001)	1.55 (0.129)	0.00	1.00	0.19
Anthesis	capitula length (mm)	43.42 (< 0.0001)	1.56 (0.1279)	0.00	0.96	1.00
	capitula width (mm)	210.59 (< 0.0001)	3.16 (0.0012)	<u>1.00</u>	1.00	1.00
	capitulum shape	196.55 (< 0.0001)	2.17 (0.0246)	<u>0.96</u>	0.99	1.00
	pistillate florets	494.67 (< 0.0001)	1.46 (0.1612)	<u>1.00</u>	0.00	0.00
	perfect florets	13.74 (< 0.0002)	0.5 (0.8734)	0.75	1.00	1.00
	total florets	252.9 (< 0.0001)	0.84 (0.5814)	<u>1.00</u>	0.00	0.95
Senescence	achene length (mm)	89.01 (< 0.0001)	5.27 (< 0.0001)	<u>1.00</u>	<u>0.71</u>	<u>1.00</u>

<sup>a</sup> = phenological stages: rosette, determined five to six weeks after seedling emergence; cauline, two weeks post stem elongation but prior to anthesis; anthesis, post anthesis but prior to maturation; senescence, post physiological maturity but prior to the anemochorous stage.

<sup>b</sup> = mean squares were derived from type I sums of squares.  $F$ -values for the random family effect were calculated by dividing mean squares by the residual error; per contra, the fixed taxon effect used the family within taxon mean squares.

<sup>c</sup> =  $\sigma^2$  components were estimated by the restricted maximum likelihood method (REML) and used to estimate the intraclass correlation coefficient according to Equation 1.

<sup>d</sup> = data neglected to converge Shapiro–Wilk’s test of normality, thus  $\sigma^2$  were normalized through natural–log transformation prior to ANOVA.

<sup>e</sup> = non–parametric (NP); normality  $H_0$  was rejected, thus ANOVA was restricted to elucidating differences among taxa.

<sup>f</sup> = specific leaf area (SLA) calculated the proportion of the total leaf area per unit fresh weight.

**Table 4.** Comparison of quantitative traits among *Conyza* taxa. Values represent the mean of five observations per each of ten sampled plants and repeated in time ( $n = 100$ ); numbers in parenthesis designate the standard error associated with means ( $\sigma_M$ ). A one-tailed sign test of intermediate versus non-intermediate was used to test the interspecific hybrid ( $F_1^H$ ) hypothesis for phenotypic intermediacy.

Stage <sup>a</sup>	Quantitative trait	<i>C. canadensis</i>	$F_1^H$	<i>C. ramosissima</i>	MSD <sup>b</sup>	$F_1^H$ different from parent	Phenotypic intermediacy
Rosette	diameter (mm)	107.09 (2.00)	76.79 (1.98)	40.18 (0.72)	10.78	both	+
	leaf number	25.53 (0.67)	87.57 (2.44)	83.42 (2.51)	5.39	<i>C. canadensis</i>	–
	leaf length (mm)	35.51 (1.03)	29.48 (0.57)	17.82 (0.35)	4.22	both	+
	leaf width (mm)	9.85 (0.27)	4.96 (0.17)	2.88 (0.07)	0.89	both	+
	leaf shape	3.66 (0.09)	6.59 (0.25)	6.50 (0.18)	0.89	<i>C. canadensis</i>	+
	leaf dentation <sup>c</sup>	3.93 (0.14)	1.39 (0.12)	0.35 (0.06)	0.43	both	+
	leaf trichomes (cm <sup>2</sup> )	1410 (55.56)	2754 (89.86)	3491 (69.82)	418	both	+
	leaf area (mm <sup>2</sup> )	157.11 (7.89)	67.17 (2.26)	26.88 (0.83)	19.23	both	+
	leaf weight (mg)	71.08 (3.61)	21.57 (0.73)	7.76 (0.27)	8.60	both	+
	SLA (mm <sup>2</sup> mg <sup>-1</sup> )	2.22 (0.01)	3.14 (0.03)	3.52 (0.03)	0.11	both	+
Cauline	stem diameter (mm)	9.01 (0.23)	2.66 (0.04)	0.99 (0.02)	0.59	both	+
	branch number <sup>c</sup>	2.07 (0.24)	21.34 (1.01)	18.79 (1.00)	2.40	<i>C. canadensis</i>	–
	leaf length (mm)	74.71 (1.76)	42.41 (1.18)	31.63 (0.65)	4.74	both	+
	leaf width (mm)	7.81 (0.24)	3.30 (0.08)	2.38 (0.05)	0.57	both	+

	leaf shape	9.89 (0.17)	13.30 (0.37)	14.01 (0.43)	1.80	<i>C. canadensis</i>	+
	leaf dentation <sup>c</sup>	3.9 (0.16)	0.61 (0.07)	0.51 (0.06)	0.38	<i>C. canadensis</i>	+
	leaf trichomes (cm <sup>2</sup> )	444 (17.50)	874 (29.06)	3237 (58.80)	260	both	+
	leaf area (mm <sup>2</sup> )	265.84 (11.10)	83.34 (3.57)	40.19 (1.28)	25.82	both	+
	leaf weight (mg)	50.58 (2.73)	13.53 (0.64)	6.73 (0.25)	4.92	both	+
	SLA (mm <sup>2</sup> mg <sup>-1</sup> )	5.53 (0.07)	6.27 (0.08)	6.12 (0.07)	0.26	<i>C. canadensis</i>	-
Anthesis	capitula length (mm)	4.90 (0.03)	4.95 (0.03)	4.50 (0.04)	0.11	<i>C. ramosissima</i>	-
	capitula width (mm)	2.01 (0.02)	1.60 (0.01)	1.58 (0.02)	0.05	<i>C. canadensis</i>	+
	capitulum shape	2.45 (0.02)	3.09 (0.01)	2.87 (0.03)	0.07	both	-
	pistillate florets	36.90 (0.54)	17.62 (0.26)	12.49 (0.27)	1.72	both	+
	perfect florets	15.68 (0.33)	14.44 (0.18)	12.84 (0.26)	1.14	both	+
	total florets	52.58 (0.77)	32.06 (0.38)	25.33 (0.41)	2.65	both	+
Senescence	achene length (mm)	0.87 (0.01)	1.13 (0.02)	1.26 (0.01)	0.06	both	+
							27:22

<sup>a</sup> = refer to Table 3 for a description.

<sup>b</sup> = minimum significant difference (MSD) estimated when ANOVA identified significant taxon effects. Mean separation was conducted by Fisher's least significant difference ( $LSD_{\alpha=0.05}$ ) when data converged the Shapiro-Wilk's assumption for normality. Else, significance was estimated by Kruskal-Wallis' analysis and *post hoc* mean separation conducted by Dunn's test (Bonferroni's method).

<sup>c</sup> = the  $\sigma^2$  of non-parametric traits was normalized by natural-log transformation prior to ANOVA.

**Table 5.** Total-sample standardized canonical coefficients (SCC) for variate 1 (Can1) and 2 (Can2), coefficients of determination ( $R^2$ ), and  $F$ -values for the 17 traits that converged Mardia's normality assumption in the evaluated *Conyza* taxa.

Stage <sup>a</sup>	quantitative trait	Can1	Can2	$R^2$	$F$ -value <sup>b</sup>
Rosette	diameter (mm)	-0.85	0.16	0.73	398.42
	leaf number	0.23	0.38	0.66	284.31
	leaf length (mm)	0.16	0.21	0.52	160.09
	leaf width (mm)	-0.62	-0.17	0.70	352.76
	leaf dentation	-0.01	-0.03	0.64	265.74
	leaf trichomes (cm <sup>2</sup> )	0.34	0.08	0.58	208.24
	SLA (mm <sup>2</sup> mg <sup>-1</sup> )	0.63	0.30	0.82	664.71
Cauline	branch number	0.16	0.24	0.52	157.62
	leaf length (mm)	-0.64	0.04	0.67	308.12
	leaf shape	0.15	-0.05	0.22	41.37
	leaf dentation	-0.48	-0.43	0.68	310.63
	leaf trichomes (cm <sup>2</sup> )	1.61	-2.40	0.91	1472.39
	SLA (mm <sup>2</sup> mg <sup>-1</sup> )	0.05	0.04	0.15	27.27
Anthesis	capitula length (mm)	0.20	0.25	0.28	58.13
	capitulum shape	0.18	0.53	0.59	214.21
	perfect florets	0.52	0.40	0.16	28.54
	total florets	-2.07	-1.08	0.82	665.79
Unweighted overall $R^2$				0.57	
Weighted by $\sigma^2$ overall $R^2$				0.77	

<sup>a</sup> = refer to Table 3 for a description.

<sup>b</sup> = univariate statistics tests the  $H_0$  that the taxa means are equal. The probability of an  $\alpha$  greater than  $F$  was  $< 0.0001$  for all traits.

**Table 6.** *R*-allele segregation in the self-pollinated *Conyza* hybrid progeny ( $F_2^H$ ) of ten families isolated from the unidirectional *C. canadensis* to *C. ramosissima* artificial cross. For backcrosses, the interspecific hybrid ( $F_1^H$ ) served as pollen donor to the *C. canadensis* ( $BC_c^H$ ) and *C. ramosissima* ( $BC_r^H$ ) parent.

Cross type	Family No.	Observed phenotype <sup>a</sup>				Expected <sup>b</sup>		$\chi^2$	$P > \chi^2$
		R	IR	S	Total	R : IR : S			
F <sub>1</sub> <sup>H</sup> self	1	4	16	7	27	6.75 : 13.5 : 6.75		1.59	0.45
	2	12	15	7	34	8.5 : 17 : 8.5		1.94	0.38
	3	10	17	5	32	8 : 16 : 8		1.69	0.43
	4	11	15	5	31	7.75 : 15.5 : 7.75		2.35	0.31
	5	6	18	2	26	6.5 : 13 : 6.5		5.08	0.08
	6	3	13	5	21	5.25 : 10.5 : 5.25		1.57	0.46
	7	8	23	4	35	8.75 : 17.5 : 8.75		4.37	0.11
	8	5	14	8	27	6.75 : 13.5 : 6.75		0.70	0.70
	9	7	17	9	33	8.25 : 16.5 : 8.25		0.27	0.87
	10	10	16	5	31	7.75 : 15.5 : 7.75		1.64	0.44
F <sub>1</sub> <sup>H</sup> self	combined <sup>c</sup>	76	164	57	297	74.25 : 148.5 : 74.25		5.67	0.06
BC <sub>c</sub> <sup>H</sup>	combined	88	70	---	158	79 : 79		2.05	0.15

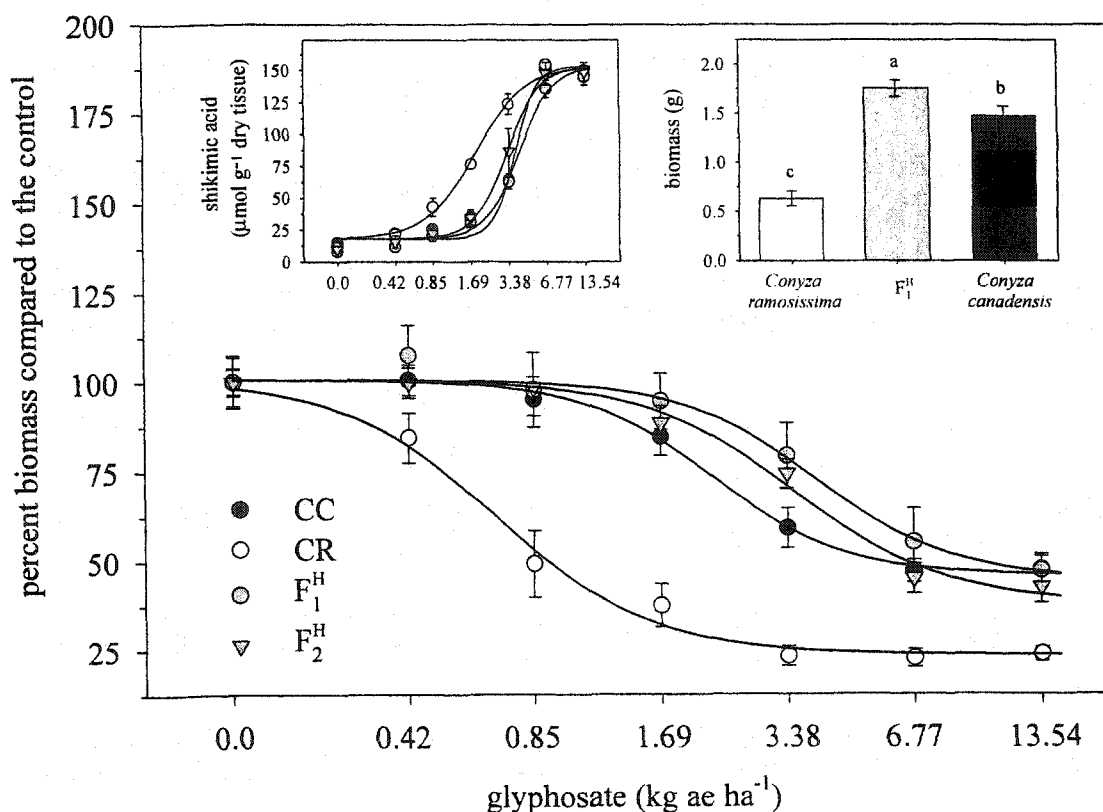
BC <sub>r</sub> <sup>H</sup>	combined	---	83	110	193	96.5 : 96.5	3.78	0.05
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<sup>a</sup> = susceptible (S) comprised individuals that developed  $\geq 70\%$  visual injury two weeks after 2 kg glyphosate ha<sup>-1</sup>; in contrast, resistant (R) and intermediate-resistant (IR) developed  $\leq 30\%$  and 31–69 % visual injury at this same rate, respectively. Both R and IR reached reproductive stage after glyphosate treatment.

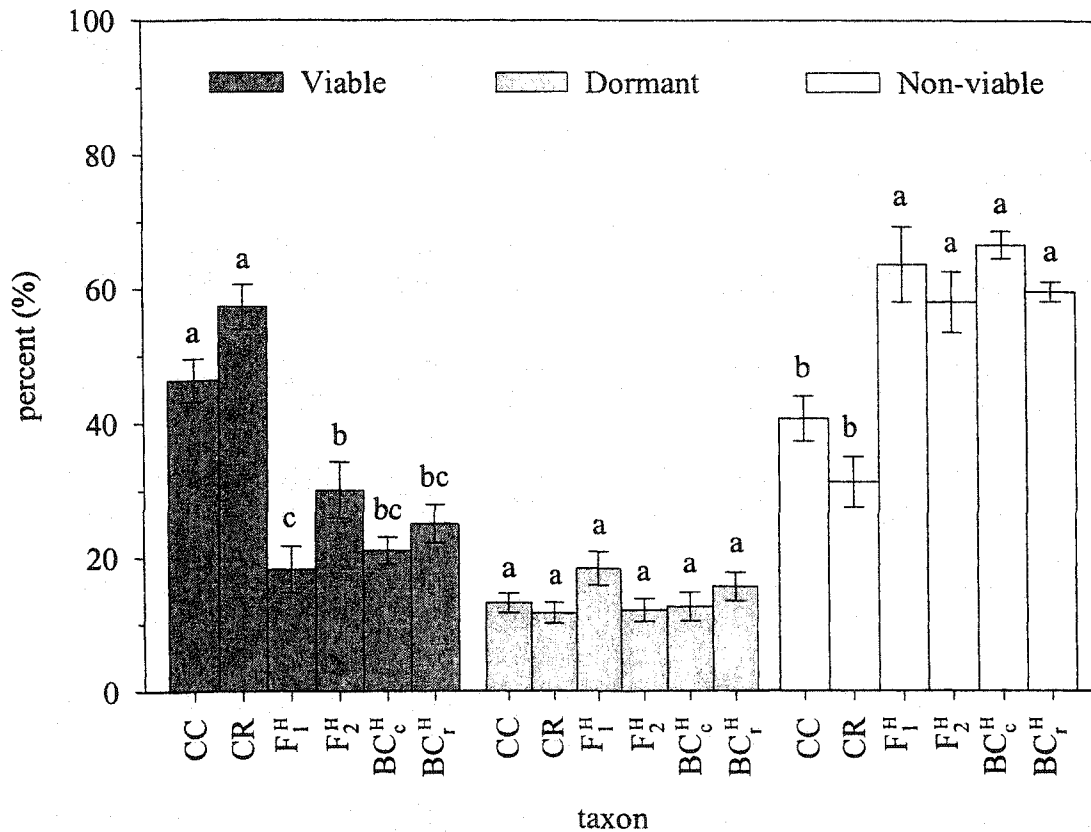
<sup>b</sup> = expected Mendelian ratios (1:2:1) for the single allele incompletely-dominant model.

<sup>c</sup> = homogeneity  $\chi^2$  test was non-significant, thus data among families was combined for the  $\chi^2$  goodness-of-fit test.



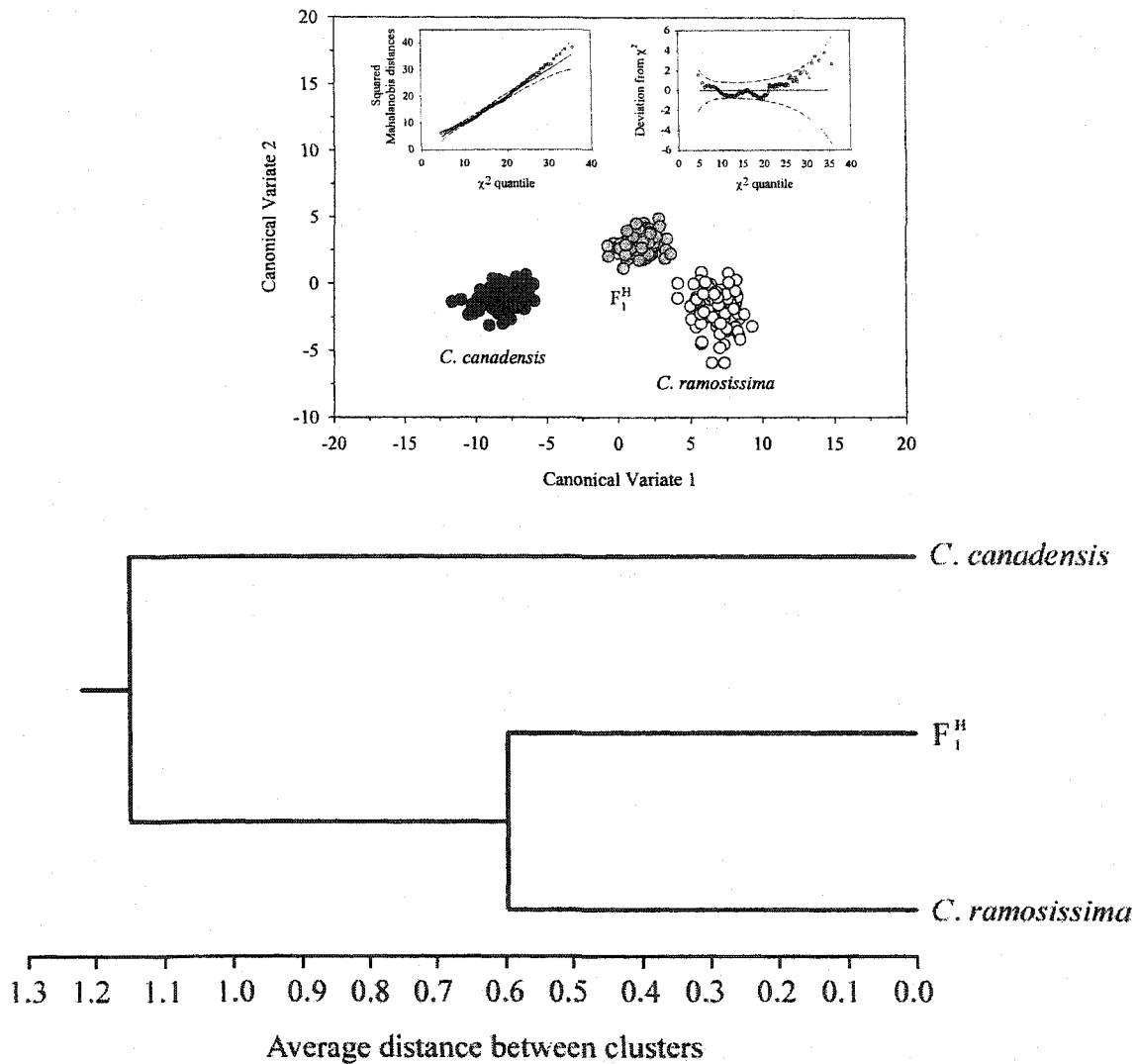


**Figure 1.** Glyphosate rate response of the *Conyza canadensis* (CC; ●), *C. ramosissima* (CR; ○), interspecific hybrid (F<sub>1</sub><sup>H</sup>; ●), and hybrid progeny (F<sub>2</sub><sup>H</sup>; ▼) populations. Data points represent the mean of four replications and two experiments ( $n = 8$ ). Three randomly selected plants per each of the ten families were used in the F<sub>1</sub><sup>H</sup> and F<sub>2</sub><sup>H</sup> rate responses. *Left insert:* endogenous shikimic acid accumulation in the tissue of treated plants. Data represents the mean of four replication and two experiments ( $n = 8$ ). *Right insert:* biomass of untreated *C. ramosissima* (white bar), F<sub>1</sub><sup>H</sup> (gray bar), and *C. canadensis* (black bar) rosettes at the 10 to 12 cm diameter stage. Bars represent the mean of ten randomly selected rosettes and determination repeated in time ( $n = 20$ ). Letters above bars represent minimum statistical differences according to Fisher's LSD for comparisons between *Conyza* taxa means ( $LSD_{\alpha=0.05} = 0.24$  g). For all graphs, extensions on bars or symbols designate the standard error associated with individual means ( $\sigma_M$ ).



**Figure 2.** Proportion of viable (dark gray bars), dormant (gray bars), and non-viable (white bars) achenes among the *Conyza canadensis* (CC), *C. ramosissima* (CR), interspecific hybrid ( $F_1^H$ ), hybrid progeny ( $F_2^H$ ), and the  $F_1^H$  backcross to *C. canadensis* ( $BC_c^H$ ) or *C.*

*ramosissima* ( $BC_r^H$ ). Bars represent the mean of two experiments comprised of achenes from the ten maternal plants or one random plants from each of the ten artificially-generated families ( $n = 20$ ); extensions above bars designate the standard error associated with individual means ( $\sigma_M$ ). Letters above bars represent minimum statistical differences according to Fisher's LSD for comparisons within viable ( $LSD_{\alpha=0.05} = 12\%$ ), dormant ( $LSD_{\alpha=0.05} = 7\%$ ), and non-viable ( $LSD_{\alpha=0.05} = 14\%$ ) achenes.



**Figure 3.** Above: multivariate discriminant function analysis of canonical variates 1 and 2 for 17 morphometric traits that converged Mardia's multivariate normality assumption. Data points represent the score of individuals ( $n=100$ ) within the *Conyza canadensis* (●), *C. ramosissima* (○), and interspecific hybrid ( $F_1^H$ ; ◐) populations. *Inserts:* chi-square ( $\chi^2$ ) quantile-quantile (Q-Q) plots of taxon observations. The continuous and dashed lines represent expected mean vectors and 75% confidence intervals for the data, respectively. Below: clustering analysis of Mahalanobis' distance matrix based on taxon averages by the unweighted pair-group method with arithmetic mean (UPGMA).

## CHAPTER 5. GENERAL CONCLUSIONS

Glyphosate (*N*-(phosphonomethyl) glycine) use in many crop environments is economical, effective, and consistent at controlling a diverse weed flora. This versatility has resulted in broad acceptance and use in domestic, agricultural, and industrial scenarios. Since the initial synthesis in the early 1970's, glyphosate has been marketed in several formulations and has helped suppress the competition of weeds in many agricultural environments, thus helping feed the growing world population. To date, the demand for glyphosate has not diminished; on the contrary, novel uses and applications are constantly being developed and increased the worldwide demand for this chemistry. Hence, many regard glyphosate as a global herbicide and the most successful agrochemical ever produced.

The many strengths associated with glyphosate include (1) broad spectrum weed control, (2) systemic activity, thus effective control of perennial plants, (3) low mammalian toxicity, (4) limited soil mobility and low risk for contamination of ground water, (5) low frequency for the evolution of resistance, and (6) economical to synthesize. Conversely, weaknesses typically associated with glyphosate include (1) antagonism effects with other herbicides, (2) loss of effectiveness due to hard water or organic matter in application water, (3) slow absorption compared to other herbicides thus potential for rain-fastness, and (4) the requirement of higher glyphosate rates to control tolerant weeds. The limited soil activity is considered a strength as it minimizes carryover and subsequent crop injury problems, yet it can also be considered a weakness since it does not provide residual weed control.

Before 1996, when glyphosate resistant crops were introduced to markets, glyphosate was primarily applied post emergence prior to row crop planting. The glyphosate resistant crop systems provided a simple, economical, and effective alternative to weed management. Yet concerns existed regarding the impact of increased glyphosate selection pressure on weed population shifts, and ultimately on the evolution of glyphosate resistant biotypes. These concerns transformed into reality in 1998 when the first glyphosate resistant weed was confirmed in Australia. Since, at least six additional weedy biotypes worldwide have been verified resistant to glyphosate, including confirmation of nine independent horseweed (*Conyza canadensis* (L.) Cronq.) populations within the United States.

Frequent reports by Midwest farmers raise concerns that glyphosate may be losing efficacy on economically important species as velvetleaf (*Abutilon theophrasti* Medicus), common lambsquarters (*Chenopodium album* L.), and common waterhemp [*Amaranthus tuberculatus* (Mq.ex DC) Sauer]. Research is needed to ascertain the potential for the evolution of glyphosate resistance in these weed species and based on the generated information, develop alternative managements strategies to mitigate the evolution of resistance. If resistance has evolved, as is the case with horseweed, research is needed to understand the potential for resistant biotypes to disperse in the agroecosystems. In addition to resistance movement through pollen and seed, interspecific hybridization represents a viable route for the dissemination of resistance among related weed species. Furthermore, between species gene flow may increase the genetic diversity of the genus and further complicate the management of resistant biotypes.

The research herein described evaluated two aspects of resistance to glyphosate: (1) investigated the potential for glyphosate resistance to evolve, and (2) elucidated the genetics of glyphosate resistance. It was demonstrated that the persistent glyphosate selection in an *A. tuberculatus* population lead to increases of resistant individuals within the population. Since the level of glyphosate resistance increased in the *A. tuberculatus* population isolated through recurrent selection, suggested that this response may have a genetic component. However, questions arise as to why glyphosate resistance in *A. tuberculatus* has not been documented in the field. First, I contend that since selection pressure in the field is affected by biotic and abiotic factors, evolution of resistance may require several cycles of selection. The seedling assay used in the selection of *A. tuberculatus* suggested that the frequency of resistant individuals within the population ranged from  $1.53 \times 10^{-3}$  to  $6.61 \times 10^{-4}$ . Thus, the rate of evolution would depend on whether these extremely rare individuals are effectively selected under field conditions. However, our data confirmed that even when two phenotypically-confirmed, resistant *A. tuberculatus* plants are crossed, the progeny demonstrated variability to glyphosate. Therefore, this variability will probably mitigate the rate of evolution and require persistent and prolonged selection for the level of glyphosate resistance to become economically relevant to farmers.

In the other component of this investigation, the potential for introgression between

horseweed and a phylogenically-related native Iowa weed was evaluated. Glyphosate resistance was successfully transferred from horseweed to the receptor species, suggesting that efforts to mitigate glyphosate resistance should not only focus on seed and pollen spread, but should include the potential for hybridization with related species. Furthermore, it was demonstrated that interspecific hybridization increased the genetic diversity in the *Conyza* genus by generating individuals with diverse phenotypes. It is speculated that under some agricultural ecosystems, these rare phenotypes may exploits specific niches and evolve into novel weed problems. Most farmers base weed management decisions on the economic return, simplicity, and effectiveness of the control tactic(s). Since most production systems depend on chemical control for weed management, farmers often rely on herbicides of similar modes of action and use them repetitively. These practices often increase the selection pressure and the potential for herbicide resistance to evolve. Management practices that favor the evolution of herbicide resistance should be combined with alternative weed control tactics, thus maintaining the efficacy of our current weed control programs and preserving the sustainability of agricultural ecosystems.

**APPENDIX 1. EVOLVED RESISTANCE TO ALS-INHIBITING  
HERBICIDES IN COMMON SUNFLOWER (*HELIANTHUS ANNUUS*),  
GIANT RAGWEED (*AMBROSIA TRIFIDA*), AND SHATTERCANE  
(*SORGHUM BICOLOR* (L.) MOENCH) IN IOWA**

A paper published in *Weed Science*<sup>1</sup>

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**Abstract**

Weed biotypes putatively resistant to ALS inhibiting herbicides were reported by Iowa farmers from 1997 to 2001. Greenhouse studies confirmed cross-resistance to triazolopyrimidine sulfonanilide (TP) and sulfonylurea (SU) herbicides in giant ragweed from Scott County, IA (Werner Farm), which corresponded to a 21 and 28 resistance to susceptibility  $GR_{50}$  (R/S) ratio to cloransulam and primisulfuron + prosulfuron, respectively. At the enzyme level, this represented a 49 and 20 fold  $I_{50}$  increase. Cross-resistance to imidazolinone (IMI) and SU herbicides was also observed in common sunflower from Cherokee, IA. Compared to a susceptible biotype, the resistant common sunflower biotype demonstrated a 36 and 43  $GR_{50}$  R/S ratio to imazethapyr and chlorimuron, respectively. Shattercane from Malvern, IA was susceptible to nicosulfuron but was resistant to imazethapyr ( $GR_{50}$  R/S ratio = 29). The woolly cupgrass biotypes from Union County, IA (Pettit Farm; Travis Farm) were reportedly resistant, but were identified susceptible to both IMI and SU herbicides. Utilizing an *in vivo* ALS assay, extractable endogenous 2,3-diketone concentrations ranged from 25 to 71 nmol g<sup>-1</sup> fresh weight for all species. Compared to susceptible biotypes, 2,3-diketone levels accumulated to at least two fold higher in treated resistant plants 120 h after herbicide application. Field history data suggested that resistance

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evolved independently in three environments where ALS inhibiting herbicides represented an important component of the selection pressure.

### Introduction

The structure of the gene encoding acetolactate synthase (ALS, EC 2.2.1.6; formerly EC 4.1.3.18) varies from nuclear-encoded operons in bacteria to plastidic monocistronic arrangements in algae (Wek et al. 1985; Reith and Munholland 1993). Mammalian systems, however, are devoid of functional ALS and thus depend on dietary intake of the essential branched-chain amino acids for survival (Whitcomb 1999). ALS inhibiting herbicides have diverse stereochemistry and lack competitive inhibition of the substrates for ALS, suggesting that inhibitory ligands bind to allosteric domains within the enzyme (Schloss et al. 1988; Durner and Böger 1991).

The low mammalian toxicity, high efficacy at extremely low concentrations, and effective control of a diverse weed flora led to the acceptance of ALS inhibiting herbicides as effective weed management tools. Nevertheless, the rapid evolution of resistance within target species diminished the efficacy of these chemistries and at present, restricts their use in several ecosystems (Whitcomb 1999). As weeds interact with environmental factors, resistance evolves through genome modification that occurs by recombination during DNA replication, random allele mutation(s), gene migration, and gene drift. The herbicide resistance evolution rate, which is proportional to the selection pressure, is maximum in environments where residual herbicides with single modes of action are applied repeatedly (Gressel and Segel 1978). Currently, most cases of herbicide resistance are endemic to countries with high-input agriculture programs and intensive and extensive production regions, specifically the United States of America (USA) and Canada (Heap 2003).

From 1997 to 2001, Iowa farmers reported cases of inadequate ALS inhibiting herbicide efficacy to common sunflower (*Helianthus annuus* L.), giant ragweed (*Ambrosia trifida* L.), shattercane [*Sorghum bicolor* (L.) Moench], and woolly cupgrass [*Eriochloa villosa* (Thunb.) Kunth]. Greenhouse and laboratory studies were conducted to evaluate the response of these biotypes to selected ALS inhibiting herbicides and to determine the proportion of resistant and susceptible individuals within the populations. Herein we report



on the evolved resistance in shattercane and cross-resistance in common sunflower and giant ragweed to ALS inhibiting herbicides.

## Materials and Methods

### Plant Material

The susceptible common sunflower (Woodruff Farm, Ames, IA), giant ragweed (Zearing, IA), shattercane (Curtiss Farm, Ames, IA), and woolly cupgrass (Atlantic, IA) biotypes were obtained from the Iowa State University weed seed collection. While the materials were originally collected from agroecosystems, records suggested that the biotypes were susceptible to ALS inhibiting herbicides. The resistant biotypes evolved in environments of selection pressure from ALS inhibiting herbicides. In 2001, a giant ragweed biotype was reported in Scott County, IA (Werner Farm) where combined glyphosate, the methyl ester of cloransulam, clethodim, and lactofen applications failed to provide adequate control (D. Friederichs, personal communication). At soybean [*Glycine max* (L.) Merr.] harvest, mature giant ragweed plants were removed from the field, dried at room temperature, and seeds stored at 5 C until utilized. The woolly cupgrass biotypes were reported in 1998 in Union County, IA (Travis Farm and Pettit Farm) (T. Cameron, personal communication). Seed from the soil and from alleged-resistant plants were collected by a local farmer prior to maize (*Zea mays* L.) harvest and stored at 5 C until utilized. The common sunflower biotype, identified north of Cherokee, IA in 1997, reportedly resisted combined applications of chlorimuron, imazethapyr, thifensulfuron, and imazaquin according to a local cooperative representative. The location was surveyed and seeds were collected from plants in four sectors of the field where suspected resistant plants had reached maturity; seeds were stored under the conditions previously indicated (J. Lee, personal communication). Seeds from the suspected resistant shattercane were collected near Malvern, IA in 1998 by a local crop consultant; these seeds were submitted to Iowa State University and stored at 5 C until utilized (A. White, personal communication).

### Germination, Growth Conditions, and Asexual Reproduction

Seeds were cleaned in an air column separator<sup>1</sup> and stored at 5 C until utilized. Conditioning comprised removing the distal-end of common sunflower, giant ragweed, and shattercane seeds with a razor blade and imbibing seeds in 0.1  $\mu\text{M}$  GA<sub>3</sub><sup>2</sup> for 24 h. Woolly cupgrass was germinated without seed conditioning. Seeds were germinated in flats with a peat:perlite:loam (1:2:1 v:v:v) soil-mix media. Upon emergence, four seedlings of woolly cupgrass and shattercane and three seedlings of common sunflower and giant ragweed were transplanted into individual 12 cm diam pots. Plants were irrigated as needed, fertilized<sup>3</sup> two wk after transplanting, and grown under natural light supplemented to a 16 h photoperiod with 600–1000  $\mu\text{mol}^{-2} \text{ m s}^{-1}$  PPFD artificial illumination. Greenhouse conditions included 28 to 35 C and 50 to 80% relative humidity (RH) during the day and 20 to 25 C and 50% RH at night.

Asexually propagated plantlets were prepared by pruning common sunflower or giant ragweed branches to three fully-expanded leaves and cutting the stem in water to prevent xylem cavitation. The cut branches were dusted with hormone<sup>4</sup> at the base, placed in silica sand<sup>5</sup>, and grown at 500–600  $\mu\text{mol}^{-2} \text{ m s}^{-1}$  PPFD and 70–80 % RH for three weeks. Plantlets that developed adventitious roots were transplanted to an autoclaved peat:perlite:loam soil-mix, fertilized weekly, and placed under a 16 h photoperiod to promote vegetative development. Asexual common sunflower and giant ragweed propagation enabled the perpetuation of genotypes, facilitated seed increase, and permitted the confirmation of resistant and susceptible genotypes non-destructively.

### Herbicide Whole-Plant Dose Responses

Treatments were applied in a CO<sub>2</sub>-powered spray chamber<sup>6</sup> delivering 187 l ha<sup>-1</sup> at 2.8 kg per cm<sup>2</sup> through an even flat flow nozzle<sup>7</sup> at 30 cm from the canopy of plants. Plants were treated with deionized water ( $\text{dH}_2\text{O}$ ) or 0.25, 0.5, 0.75, 1, 2, 4, 16, or 64 times the herbicide rate recommended by the manufacturer at the plant height indicated in Table 1. Treatment efficacy was evaluated two wk after herbicide application by estimating the percent foliage damage of test plants compared to the  $\text{dH}_2\text{O}$ -treated control plants. Asymptomatic plants were ascribed 0% injury while completely necrotic plants received a

100% herbicide injury rating. In addition, biomass was determined by cutting plants at the soil surface, drying the tissue at 35 C for 48 h, and determining the weight of individual samples.

### **Assessment of Resistance Frequency Within the Populations**

Preliminary assessments indicated that the weed populations consisted of individuals with either a resistant or susceptible phenotype to ALS inhibiting herbicides. Thus, the proportion of resistant, intermediate-resistant, and susceptible (R:IR:S) individuals within the population was estimated by growing plants as previously indicated and treating the target species with four times the rate recommended by the manufacturer (Table 1). Fifty plants per biotype were treated; herbicide efficacy was evaluated two wk after application and experiments were repeated once in time. Previous greenhouse determinations confirmed that plants which developed  $\leq 10\%$  visual injury two wk after herbicide application, demonstrated growth rates analogous to their corresponding  $\text{H}_2\text{O}$ -treated control plants. Therefore, R, IR, and S phenotypes were classified as those individuals that developed  $\leq 10\%$ , 11–60% and  $> 60\%$  visual herbicide injury at evaluation, respectively. The S phenotypes were effectively controlled at four times the recommended herbicide rate, however R and IR phenotypes resisted the herbicide application and reached reproductive stage.

### ***In vivo* ALS Assay**

#### ***Selection of Plant Materials for the in vivo ALS Assay***

Because populations differed in the proportion of individuals R and S to ALS inhibiting herbicides, homogenously-resistant populations were isolated by treating shattercane plants with  $280 \text{ g ae ha}^{-1}$  imazethapyr, common sunflower with  $17 \text{ g chlorimuron plus } 140 \text{ g ai ha}^{-1}$  imazethapyr, and giant ragweed with a combination of 20, 59, and  $35 \text{ g ai ha}^{-1}$  prosulfuron, the methyl ester of primisulfuron, and cloransulam, respectively. These herbicide rates corresponded to four times the rate recommended by the manufacturer for the target species (Table 1). Two wk after application, plants that demonstrated a R phenotype were allowed to self or cross-pollinate and the progeny of these selected plants used in the *in*

*vivo* ALS assay. Efficacy assessment with ALS inhibiting herbicides demonstrated that the selected populations contained negligible frequencies of S individuals (data not shown).

#### *2-Acetolactate Accretion as a Function of Time and Herbicide Concentration*

Plants grown under the greenhouse conditions previously described were pruned to three fully expanded leaves at the plant height indicated in Table 1. Shattercane plants were not pruned. Furthermore, the stems of pruned plants were cut diagonally at the soil surface and plants placed immediately in  $\text{dH}_2\text{O}$  to mitigate xylem cavitation. Finally, four plants per treatment were placed in 13 x 100 mm culture tubes with a 1:1 (v:v) solution of 250  $\mu\text{M}$  1,1-cyclopropanedicarboxylic acid (CPCA)<sup>8</sup> plus 250  $\mu\text{M}$  2-methylphosphinoyl-2-hydroxyacetic acid (HOE 704)<sup>9</sup> and  $10^{-1}$ , 1,  $10^1$ ,  $10^2$ ,  $10^3$ ,  $10^4$ , or  $10^5$  times the herbicide rate indicated in Table 1. Positive and negative controls corresponded to treatments with the CPCA:HOE 704 (double-inhibitor) solution alone or  $\text{dH}_2\text{O}$ , respectively. Monocotyledonous and dicotyledonous species received 4 and 10 ml of the double-inhibitor:herbicide solution, respectively, and were grown under a shade fabric transmitting  $500\text{--}600\ \mu\text{mol}^{-2}\ \text{m s}^{-1}$  PPFD for 72 h to allow concomitant inhibition of ALS and ketol-acid reductoisomerase (KARI, EC 1.1.1.86). Depending on the species, the inhibitor solution was absorbed within 24 to 48 h, thereafter Hoagland's No. 2 basal salt media<sup>8</sup> containing 100 mM L-alanine was provided as needed to sustain plant growth. For the time analysis of 2-acetolactate accumulation as a function of herbicide application, plants were treated as indicated, incubated in a 1:1 (v:v) solution of the double-inhibitor:manufacturer's recommended herbicide rate, and harvested 0, 12, 24, 48, 72, 96, and 120 h after incubation (Table 1).

#### **Extraction of 2-Acetolactate and Determination by Westerfeld's Test**

2-Acetolactate was estimated indirectly through its decarboxylated byproduct following a modified Westerfeld's test (Westerfeld 1945). Plants were removed from culture tubes, weighed, placed in 50 ml centrifuge tubes, and frozen at  $-20\ \text{C}$  for 24 h to promote cell lysis. The tissue was thawed in a 1:5 (w:v) ratio of fresh weight per ml of  $\text{dH}_2\text{O}$ , the tube vortexed for 5 min, and allowed to rest at  $5\ \text{C}$  for 24 h. A 500  $\mu\text{l}$  aliquot of plant extract was mixed with 50  $\mu\text{l}$  of 6 N  $\text{H}_2\text{SO}_4$  and incubated in the dark at  $60\ \text{C}$  for 15 min to decarboxylate 2-acetolactate to (*R*)-3-hydroxy-2-butanone [(*R*)-acetoin]. Samples were kept in capped 2 ml centrifuge tubes to

minimize (*R*)-acetoin volatilization. (*R*)-acetoin was then oxidized to 2,3-butanedione (diacetyl) by adding 1000  $\mu$ l of 40 mM creatine<sup>8</sup> plus 350 mM  $\alpha$ -naphthol<sup>8</sup> dissolved in 2 N NaOH and incubating at 60 C for 30 min. The diacetyl chromophore had a 420–620 nm absorbance range, 520 nm absorbance maximum ( $\lambda_{max}$ ), and  $2.36 \times 10^4$  l mol<sup>-1</sup> cm<sup>-1</sup> molar absorptivity ( $\epsilon_{520}$ ) at 25 C and pH of 13.5. Because the ALS reaction synthesizes two butanoate products, both interacting with Westerfeld's reagents and creating diacetyl and acetyl propionyl (2,3-pentanedione) chromophores,  $A_{520}$  data was converted to mole equivalents utilizing the molecular weight of 86.09 for diacetyl, the predominant chromogenic 2,3-diketone species in the assay solution. Therefore, the spectrophotometer<sup>10</sup> calibrated with diacetyl<sup>8</sup> at 0.05–10  $\mu$ g ml<sup>-1</sup> reported  $A_{520}$  data as 2,3-diketones nmol g<sup>-1</sup> fresh weight.

### Statistical Analysis

The dose response experiments were arranged as a randomized complete block design (RCBD) with four blocks and repeated once in time. Visual injury data were subjected to analysis of variance (ANOVA) and means separated by Fisher's least significant difference (LSD) test at an  $\alpha$  of 0.05 (SAS 2000). Biomass and *in vivo* ALS activity data were tested to several non-linear regression models and the fit assessed by lack-of-fit (LOF) tests and graphically by the distribution of residuals (SPSS 2002). Growth reduction 50% ( $GR_{50}$ ) and enzyme inhibition 50% ( $I_{50}$ ) values, defined as the herbicide dose that inhibited plant growth or apparent *in vivo* ALS activity by 50%, were calculated by PROC NLIN based on the log-logistic model (Seefeldt et al. 1995). In addition, visual injury data were converted to a binary format following the descriptor for R ( $\leq 60\%$ ) and S ( $> 60\%$ ) phenotypes and analyzed with a modified Newton-Raphson algorithm (SAS 2000). The herbicide dose that inflicted 50% mortality in the population ( $LD_{50}$ ) was estimated by PROC PROBIT.

## Results and Discussion

### Species Response to Selected ALS Inhibiting Herbicides

#### *Giant Ragweed*

Giant ragweed resistance to ALS inhibiting herbicides evolved in a maize-soybean rotation system where herbicides with different modes of action, including pendimethalin,

trifluralin, and flumetsulam, were applied pre emergence (PRE) during soybean production and acetochlor and atrazine during maize (Table 2). The Scott County, Iowa (Werner Farm) giant ragweed biotype was effectively controlled post emergence (POST) with ALS inhibiting herbicides for at least 2 yr prior to the decline in efficacy reported in 2001. The level of resistance in 1999 was probably too low to cause a concern to the farmer; however, seed from giant ragweed plants surviving the application of two residual ALS inhibiting herbicides could have impacted the relative R/S ratio in the field. Effective weed control was attained in 2000 with nicosulfuron + rimsulfuron + atrazine at 13, 13 and 800 g ai ha<sup>-1</sup>, respectively, plus diglycolamine salt of dicamba at 0.28 kg ai ha<sup>-1</sup>. In 2001, weed control was based on a 3.3 kg ai ha<sup>-1</sup> pendimethalin PRE and 77 g ai ha<sup>-1</sup> flumetsulam and 0.5 kg ae ha<sup>-1</sup> glyphosate POST applications. The resulting unsatisfactory control prompted sequential applications of 17 g ha<sup>-1</sup> cloransulam plus 87 g ai ha<sup>-1</sup> clethodim and 17 g cloransulam plus 70 g ha<sup>-1</sup> lactofen, four and six wk after the initial application. At harvest, approximately 20% of the field was infested with giant ragweed resistant to ALS inhibiting herbicides. Greenhouse studies confirmed that the Werner Farm biotype was cross-resistant to triazolopyrimidine sulfonanilide (TP) and sulfonylurea (SU) herbicides (Figure 1). Concomitantly, visual estimates indicated that plants were consistently less injured and that the population demonstrated an enhanced survival to cloransulam and primisulfuron + prosulfuron compared to the susceptible Zearing, IA biotype (Figure 1). The herbicide-treated IR giant ragweed phenotype had a shorter plant internode length, activated lateral branch growth, developed deformed-chlorotic apical leaves, and demonstrated lower growth rates compared to the treated R or untreated S phenotypes. Phenotypic analysis indicated that the Werner Farm population contained at least 88% R and IR individuals to cloransulam and primisulfuron + prosulfuron (Table 3). Log-logistic analysis estimated *GR*<sub>50</sub> value increases of 21-fold to cloransulam and 28-fold to primisulfuron + prosulfuron compared to the S biotype (Table 4). Concurrently, *LD*<sub>50</sub> and *I*<sub>50</sub> values for the Werner Farm population were 79- and 49-fold higher to cloransulam and 102- and 20-fold to primisulfuron + prosulfuron compared to the Zearing, IA population. Utilizing the *in vivo* ALS assay, negative and positive controls permitted the quantification of 50 nmol minimum and 600 nmol maximum extractable 2,3-diketone levels per g of fresh weight for giant ragweed.

Additionally, the ALS assay permitted confirmation of cross-resistance in giant ragweed. The resistant giant ragweed biotype accumulated at least twice the level of 2,3-diketones compared to the susceptible biotype at 120 h of cloransulam or primisulfuron + prosulfuron application (Figure 1). This suggested that ALS of the Werner Farm biotypes was more active at equimolar herbicide concentrations compared to ALS of the Zearing, IA biotypes. We cannot conclude, however, that resistance was attributable to a polymorphic *ALS* as differences in herbicide translocation, metabolism, or sequestration could have contributed to the differential inhibition of ALS in resistant and susceptible biotypes.

Previous research ascribed the interspecific variation of *Ambrosia* species to ALS inhibiting herbicides to reduced imazethapyr translocation and enhanced glycosilation in common ragweed (*Ambrosia artemisiifolia* L.) compared to giant ragweed (Ballard et al. 1995, 1996). Field trials with cloransulam from 1994 until its introduction in 1998 described the herbicide as effective on diverse dicotyledonous species (Franey and Hart 1999; Leif et al. 2000). Nonetheless, at least 30 common and giant ragweed biotypes were reported resistant to cloransulam during the first year of that herbicide's commercialization (Patzoldt et al. 2001). While no absorption, translocation, or metabolism studies of ALS inhibiting herbicides were conducted, cross-resistance in giant ragweed was attributed to an altered *ALS* (Patzoldt and Tranel 2002). Further complicating the containment of herbicide resistance in giant ragweed is the capacity for wind pollination and introgression with other *Ambrosia* species (Bassett and Crompton 1982). In 2000, Iowa farmers reported fifty sites with giant ragweed biotypes resistant to ALS inhibiting herbicides, infesting approximately 400 ha of maize and soybean fields (R.G. Hartzler, personal communication).

### *Woolly Cupgrass*

The suspected resistant Union County Iowa (Travis and Pettit Farm) woolly cupgrass biotypes evolved in a continuous maize system, coexisting in a field with a 25 yr history of high-density grass weeds. The farmer based weed management on a pendimethalin, atrazine, and metolachlor PRE program followed by POST applications of nicosulfuron and mechanical control (Table 2). Nicosulfuron at 35 to 52 g ai ha<sup>-1</sup> was applied once or twice each growing season from 1989 to 1997 until unacceptable control by nicosulfuron was

reported in 1998. Applications under controlled conditions confirmed that both Travis Farm and Pettit Farm biotypes were homogenously susceptible to the manufacturer's recommended rates for imidazolinone (IMI) and SU herbicides (Table 3). In addition,  $GR_{50}$ ,  $LD_{50}$ , and visual injury values were similar to those of the susceptible Atlantic, IA biotype (data not shown). We therefore attributed the unacceptable control reported by the farmer to either an application problem or to the weed shift that occurred due to the over-reliance on ALS inhibiting herbicides resulting in a high woolly cupgrass population density in the field. Woolly cupgrass tolerance to ALS inhibiting herbicides was related to  $P_{450}$ -mediated hydroxylation (Hinz et al. 1997). Under field conditions, POST applications of ALS inhibiting herbicides rarely provide complete woolly cupgrass control and often require cultivation or the application of non-ALS inhibiting herbicides to maximize efficacy (Young and Hart 1999; Mickelson and Harvey 2000). Therefore, herbicide application timing is a critical factor in maximizing efficacy and allowing for woolly cupgrass management through seed bank depletion (Rabaey et al. 1996; Buhler and Hartzler 2001).

### *Common Sunflower*

The common sunflower biotype allegedly resistant to ALS inhibiting herbicides evolved in a soybean-maize rotation (Table 2). Weed management of the Cherokee, IA field relied on PRE applications of acetochlor + atrazine in maize and trifluralin in soybean, followed by POST applications of ALS inhibiting herbicides. Adequate weed control was achieved with acetochlor + atrazine plus nicosulfuron in 1995. However, POST application of chlorimuron at  $13 \text{ g ha}^{-1}$  and fluazifop + fenoxaprop at 2 and  $0.56 \text{ kg ai ha}^{-1}$  provided unsatisfactory common sunflower control in 1996. To meet weed control expectations, the farmer therefore followed with sequential applications of 70.1, 6.5, and  $2.2 \text{ g ha}^{-1}$  of imazethapyr, chlorimuron, and the methyl ester of thifensulfuron, respectively. Nevertheless, common sunflower plants in four regions of the field reportedly survived these applications. The following yr, soybeans received a  $32 \text{ kg ha}^{-1}$  PRE application of trifluralin followed by  $13 \text{ g ha}^{-1}$  imazethapyr and  $1.2 \text{ g ai ha}^{-1}$  thifensulfuron POST. In addition, the surviving common sunflower received split applications of  $13 \text{ g ha}^{-1}$  chlorimuron and  $73 \text{ g ha}^{-1}$  imazaquin resulting only in limited common sunflower control in the 1996 problematic



areas. Greenhouse evaluations of the Cherokee, IA biotype indicated that common sunflower plants demonstrated less herbicide injury and had enhanced survival to chlorimuron and imazethapyr compared to the Ames, IA (Woodruff Farm) biotype (Figure 2). This divergence in efficacy translated to an estimated  $GR_{50}$  and  $LD_{50}$  value increase of 43- and 97-fold for chlorimuron and 36-fold and 77-fold for imazethapyr, respectively (Table 4).

At the enzyme level, these differences represented a 79-fold increase for chlorimuron and 164-fold for imazethapyr. When incubated with 10 nM chlorimuron or 100 nM imazethapyr, both resistant and susceptible biotypes demonstrated a linear 2,3-diketone increase within 24 hr of herbicide application (Figure 2). This response followed a lag-phase at approximately 48 hr and resulted in a two-fold 2,3-diketone increase at 120 h for the Cherokee, IA biotype. These results suggested that while the response to chlorimuron and imazethapyr was heterogeneous (Table 3), the Cherokee, IA common sunflower biotype was cross-resistant to both IMI and SU herbicides. The treated IR common sunflower phenotype demonstrated chlorotic-necrotic apices and lower growth rates compared to the treated R or untreated S phenotypes.

Common sunflower resistance to ALS inhibiting herbicides has evolved in several locations within the Midwest USA. In Kansas, resistance and cross-resistance to IMI and SU herbicides in common sunflower biotypes was attributed to an altered *ALS*, however some differences in herbicide absorption and translocation were reported (Al-Khatib et al. 1998; Baumgartner et al. 1999). Similarly, differences in herbicide absorption and translocation observed in a cross-resistant biotype from South Dakota indicated that these factors, in addition to a polymorphic *ALS*, collectively contribute to the expression of resistance in common sunflower (White et al. 2002). The trait for resistance imposed no apparent physiological penalty on common sunflower plants (Marshall et al. 2001), suggesting that while resistant phenotypes contained an altered *ALS*, the dynamics of the branched-chain amino acid pathway occurred at rates comparable to wild-type common sunflower plants. While management of common sunflower biotypes resistant to ALS inhibiting herbicides was attempted with combinations of ALS inhibiting, triazine, diphenyl ether, and benzothiadiazole herbicides, consistent control and better economic returns were

obtained with glyphosate in glyphosate resistant crop systems (Al-Khatib et al. 2000; Allen et al. 2001).

### *Shattercane*

Similar to the woolly cupgrass biotypes, the alleged resistant Malvern, IA shattercane biotype evolved in a continuous maize monoculture system (Table 2). Weed management in this production system comprised PRE acetochlor applications coupled with POST ALS inhibiting herbicides. While herbicides with different modes of action were part of the management program, the farmer relied primarily on POST applications of ALS inhibiting herbicides for weed control from 1995 to 1998. As part of the herbicide rotation program in 1998, acetochlor was replaced by dimethenamid at 1.5 kg ai ha<sup>-1</sup> PRE followed by POST applications of 46 and 15 g ha<sup>-1</sup> imazethapyr and imazapyr plus 0.8 kg ha<sup>-1</sup> dicamba at 15 to 20 cm plant height. Surviving shattercane plants were treated with 52 g ha<sup>-1</sup> primisulfuron or 70 g ha<sup>-1</sup> nicosulfuron, which provided only limited control. Assessment under controlled conditions indicated that nicosulfuron control of the Malvern, IA biotype was not different from the susceptible biotype (Table 3). The lack of efficacy of nicosulfuron in the field was therefore attributed to the fact that shattercane plants were treated past the 30 cm plant height recommended by the manufacturer. Nevertheless, the Malvern, IA shattercane population demonstrated an enhanced survival and lower herbicide injury to imazethapyr compared to the susceptible Curtiss Farm biotype, which translated to a 29-fold  $GR_{50}$  and 34-fold  $I_{50}$  increase to imazethapyr (Figure 3; Table 4). Accumulation of 2,3-diketones in response to 1000 nM imazethapyr followed a linear response and resulted in a three-fold difference at 120 h for the Malvern, IA biotype (Figure 3). PROBIT analysis estimated that 3.3 kg ha<sup>-1</sup> free acid of imazethapyr were required to inflict 50% mortality in the Malvern, IA population, compared to 0.028 kg ha<sup>-1</sup> for the Curtiss Farm population (Table 4). Contrary to what was observed in giant ragweed and common sunflower, no IR phenotype to ALS inhibiting herbicides was identified in the Malvern, IA shattercane population (Table 3).

Primisulfuron resistant shattercane was first reported in Nebraska in 1994 (Anderson et al. 1998a). The primary resistance mechanism was ascribed to an altered *ALS* with a 1.8-fold reduction in affinity to pyruvate. In addition, the Nebraska shattercane biotype

demonstrated a lower herbicide absorption rate compared to the susceptible biotype (Anderson et al. 1998b). Cross-resistance to SU and IMI herbicides was also reported in other shattercane accessions within Nebraska (Lee et al. 1999). Genetic analysis identified that the alleles for resistance to primisulfuron resided in linked loci in the chromosome and inheritance followed a dominant, incomplete dominant, or additive genetic model. These results suggested independent evolution events for the three shattercane biotypes resistant to ALS inhibitor herbicides. Importantly, introgression of herbicide resistance genes within the genus *Sorghum* may complicate the management of herbicide resistant shattercane biotypes through conventional chemical practices (Smeda et al. 2000).

Whole-plant dose responses and *in vivo* ALS assay permitted the confirmation of resistance to ALS inhibiting herbicides in three Iowa weed populations. In addition, field history data suggested that these independent events evolved in environments where ALS inhibiting herbicides represented an important component of the selection pressure. Farmers typically base weed management decisions on the economic return and simplicity of the control tactic(s). However, management practices that favor the evolution of herbicide resistance should be combined with alternative weed control tactics, thus maintaining the efficacy of our current weed control programs and preserving the sustainability of agricultural ecosystems.

#### Source of Materials

- <sup>1</sup> Seedburo Equipment Company, 1022 W Jackson Boulevard, Chicago, IL 60607.
- <sup>2</sup> 90% (+)-gibberellic acid, Fisher Scientific, 2000 Park Lane Drive, Pittsburgh, PA 15275.
- <sup>3</sup> Miracle Grow Excell, Scott-Sierra Co., 14111 Scottslawn Road, Marysville, OH 43041.
- <sup>4</sup> Rootone, TechPac LLC., P.O. Box 24830, Lexington, KY 40504.
- <sup>5</sup> Unimin Corp. 258 Elm Street, New Canaan, CT 06840.
- <sup>6</sup> Model SB5-66, DeVries Manufacturing, Route 1 Box 184, Hollandale, MN 56045.
- <sup>7</sup> 80015-E, TeeJet Spraying Systems, P.O. Box 7900, Wheaton, IL 60189-7900.
- <sup>8</sup> Sigma-Aldrich Corporation, 3050 Spruce Street, Saint Louis, MO 63103.
- <sup>9</sup> Hoechst AG, D-6230 Frankfurt 80, Postfach 80 03 20, Germany.
- <sup>10</sup> Lambda 18 UV/Vis Spectrometer, Perkin-Elmer, 710 Bridgeport Avenue, Shelton, CT 06484.

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**Table 1.** Description of herbicides, plant height at application, and recommended rates (1X) for giant ragweed (AMBTR), woolly cupgrass (ERBVI), common sunflower (HELAN), and shattercane (SORVU) whole-plant dose response and *in vivo* ALS assay studies.

Species	Common name <sup>a</sup>	Family <sup>b</sup>	Height (cm)	1X rates	
				g ha <sup>-1</sup>	nM
AMBTR	cloransulam	TP	12	17.4 <sup>c</sup>	10
	primisulfuron + prosulfuron	SU	12	29.6 + 9.8 <sup>c</sup>	10 + 3.7
ERBVI	imazethapyr + imazapyr	IMI	5	46.5 + 15.5 <sup>d</sup>	600 + 222
	nicosulfuron	SU	5	51.9 <sup>e</sup>	10
HELAN	imazethapyr	IMI	10	70.1 <sup>f</sup>	100
	chlorimuron	SU	12	8.6 <sup>g</sup>	10
SORVU	imazethapyr	IMI	15	70.1 <sup>f</sup>	1000
	nicosulfuron	SU	15	51.9 <sup>g</sup>	10

<sup>a</sup> = cloransulam, FirstRate<sup>®</sup>; primisulfuron + prosulfuron, Spirit<sup>®</sup>; imazethapyr + imazapyr, Lightning<sup>®</sup>; nicosulfuron, Accent<sup>®</sup>; imazethapyr, Pursuit<sup>®</sup>; chlorimuron, Classic<sup>®</sup>.

<sup>b</sup> = IMI, imidazolinone; SU, sulfonylurea; TP, triazolopyrimidine sulfonamide.

<sup>c</sup> = plus 1.25 % v:v crop oil concentrate.

<sup>d</sup> = plus 0.25 % v:v non-ionic surfactant and 1.5 % w:v ammonium sulfate.

<sup>e</sup> = plus 1.0 % v:v crop oil concentrate and 1.2 % w:v ammonium sulfate.

<sup>f</sup> = plus 1.25% v:v crop oil concentrate and 1.4 % w:v ammonium sulfate.

<sup>g</sup> = plus 1.0 % v:v crop oil concentrate.

**Table 2.** Field history leading to the evolution of resistance to ALS inhibiting herbicides in giant ragweed (AMBTR), woolly cupgrass (ERBVI), common sunflower (HELAN), and shattercane (SORVU). Herbicides with activity on the target species are depicted in italics.

Species	Biotype	Location	Year	Crop <sup>a</sup>	Weed control history <sup>b</sup>
AMBTR	Werner Farm	Scott Co, Cleona Township, Section 14	1996	—	No prior information available
			1997	soybean	pendimethalin (PRE), <i>sodium salt of fomesafen</i> (POST)
			1998	maize	acetochlor + <i>atrazine</i> (PRE)
			1999	soybean	trifluralin + <i>flumetsulam</i> tankmix (PRE), <i>cloransulam</i> + clethodim tankmix (POST)
			2000	maize	nicosulfuron + <i>rimsulfuron</i> + <i>atrazine</i> (POST), <i>diglycolamine salt of dicamba</i> (POST)
			2001	soybean	pendimethalin + <i>flumetsulam</i> + <i>glyphosate</i> tankmix (PRE), <i>cloransulam</i> + clethodim tankmix (POST), <i>cloransulam</i> + <i>lactofen</i>
ERBVI	Travis/Pettit Farm	Union Co, Lincoln Township, Section 29	1988	—	No prior information available
			1989–1995	maize	<i>pendimethalin</i> (PRE), <i>nicosulfuron</i> (POST), cultivation when needed
			1996–1998	maize	<i>atrazine</i> + <i>metolachlor</i> tankmix (PRE), <i>nicosulfuron</i> (POST), <i>nicosulfuron</i> (POST)



HELAN	Cherokee, IA	Cherokee Co, Cherokee Township, Section 22	1994	—	No prior information available
			1995	maize	acetochlor + <i>atrazine</i> (PRE), nicosulfuron (POST), nicosulfuron (POST)
			1996	soybean	<i>chlorimuron</i> + fluazifop + fenoxaprop tankmix (POST), <i>imazethapyr</i> + <i>thifensulfuron</i> tankmix (POST)
			1997	soybean	trifluralin (PRE), <i>chlorimuron</i> + <i>imazethapyr</i> tankmix (POST), <i>thifensulfuron</i> + <i>imazaquin</i> tankmix (POST)
SORVU	Malvern, IA	Mills Co, Deer Creek Township, Section 32	1994	—	No prior information available
			1995	maize	<i>nicosulfuron</i> + <i>atrazine</i> + bromoxynil tankmix (POST)
			1996	IMI maize	<i>acetochlor</i> (PRE), <i>imazethapyr</i> + <i>atrazine</i> (POST)
			1997	IMI maize	<i>acetochlor</i> (PRE), <i>imazethapyr</i> + dicamba tankmix (POST)
			1998	IMI maize	<i>dimethenamid</i> (PRE), <i>imazethapyr</i> + imazapyr + dicamba tankmix (POST), <i>primisulfuron</i> (POST) or <i>nicosulfuron</i> (POST)

<sup>a</sup> = maize (*Zea mays* L.), conventional maize; soybean [*Glycine max* (L.) Merr.], conventional soybean. Abbreviation: IMI maize, IMI-resistant maize.

<sup>b</sup> = (PRE), pre-emergence; (POST), post-emergence.

**Table 3.** Frequency of resistant (R), intermediate-resistant (IR) and susceptible (S) phenotypes to ALS inhibiting herbicides within the evaluated giant ragweed (AMBTR), woolly cupgrass (ERBVI), common sunflower (HELAN), and shattercane (SORVU) populations.

Species	Biotype	Herbicide	R:IR:S <sup>a</sup>	Phenotype
AMBTR	Werner Farm	cloransulam	10:78:12	resistant
		primisulfuron + prosulfuron	70:18:12	resistant
	Zearing, IA	cloransulam	0:0:100	susceptible
		primisulfuron + prosulfuron	0:0:100	susceptible
ERBVI	Travis/Pettit Farm	imazethapyr + imazapyr	0:0:100	susceptible
		nicosulfuron	0:0:100	susceptible
	Atlantic, IA	imazethapyr + imazapyr	0:0:100	susceptible
		nicosulfuron	0:0:100	susceptible
HELAN	Cherokee, IA	imazethapyr	80:11:9	resistant
		chlorimuron	82:10:8	resistant
	Woodruff Farm	imazethapyr	0:0:100	susceptible
		chlorimuron	0:0:100	susceptible
SORVU	Malvern, IA	imazethapyr	100:0:0	resistant
		nicosulfuron	0:0:100	susceptible
	Curtiss Farm	imazethapyr	0:0:100	susceptible
		nicosulfuron	0:0:100	susceptible

<sup>a</sup> = The susceptible (S) phenotype comprised individuals with > 60% visual injury two wk after application of four times the herbicide rate recommended by the manufacturer. Resistant (R) and intermediate-resistant (IR) phenotypes comprised plants with  $\leq 10\%$  and 11–60% visual herbicide injury, respectively. The herbicide-treated R phenotype demonstrated growth rates equivalent to untreated control plants; but both R and IR phenotypes reached reproductive stage. Data represents the combination of two experiments each with 50 individual observations.

**Table 4.** Whole plant dose response summary and *in vivo* ALS assay parameters for the resistant and susceptible giant ragweed (AMBTR), common sunflower (HELAN), and shattercane (SORVU) biotypes. Numbers in parenthesis that proceed  $GR_{50}$ ,  $LD_{50}$ , or  $I_{50}$  values designate 95% confidence intervals associated with the estimated parameter.

Species	Herbicide	Biotype	$GR_{50}^a$	$LD_{50}^b$	$I_{50}^c$	$RS_{GR:LD:I}^d$
AMBTR	cloransulam	Werner Farm	180.2 (51.1–309.4)	339.5 (183.1–887.6)	39.3 (19.4–59.2)	21:79:49
		Zearing, IA	8.6 (4.5–12.6)	4.3 (0.8–6.7)	0.8 (0.3–1.2)	
	primisulfuron + prosulfuron	Werner Farm	204.3 (65.3–343.3)	686.3 (360.6–1387.9)	14.3 (9.2–19.4)	28:102:20
		Zearing, IA	7.4 (3.8–10.9)	6.7 (0.8–10.7)	0.7 (0.4–1.1)	
HELAN	imazethapyr	Cherokee, IA	913.8 (216.8–1610.8)	1737.5 (853.5–3293.8)	7301.0 (2262.9–12339.1)	36:77:164
		Woodruff Farm	25.3 (15.3–35.2)	22.5 (8.3–32.8)	44.6 (21.5–67.6)	
	chlorimuron	Cherokee, IA	95.9 (19.4–172.3)	282.9 (145.5–726.2)	1038.3 (472.9–1603.8)	43:97:79
		Woodruff Farm	2.24 (1.41–3.06)	2.92 (1.54–3.95)	13.1 (6.7–19.4)	
SORVU	imazethapyr	Malvern, IA	740.2 (296.4–1184.1)	3315.0 (1660.9–17589.2)	5265.1 (2505.3–8024.9)	29:117:34

	Curtiss Farm	25.1 (19.8–30.4)	28.3 (18.5–36.6)	155.5 (78.9–232.1)	
nicosulfuron	Malvern, IA	20.1 (14.7–25.6)	34.6 (25.7–43.6)	4.1 (1.4–6.8)	
					1.4:1.6:1.3
	Curtiss Farm	14.8 (9.7–19.9)	22.2 (15.0–28.5)	3.1 (0.7–5.6)	

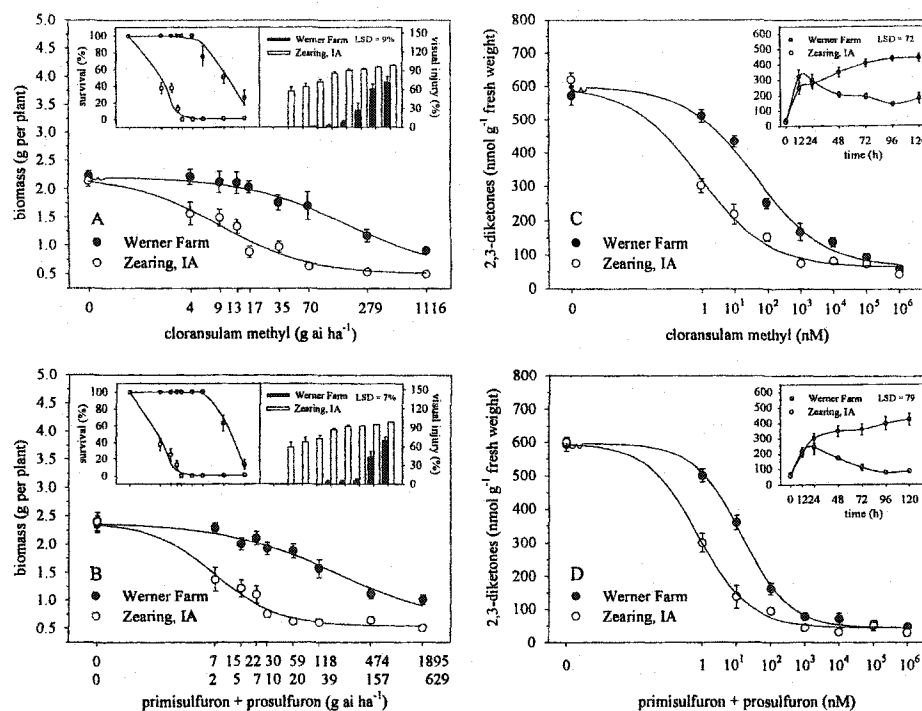
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<sup>a</sup> = herbicide dose in g ai ha<sup>-1</sup> that reduced biomass accumulation in the target species by 50%.

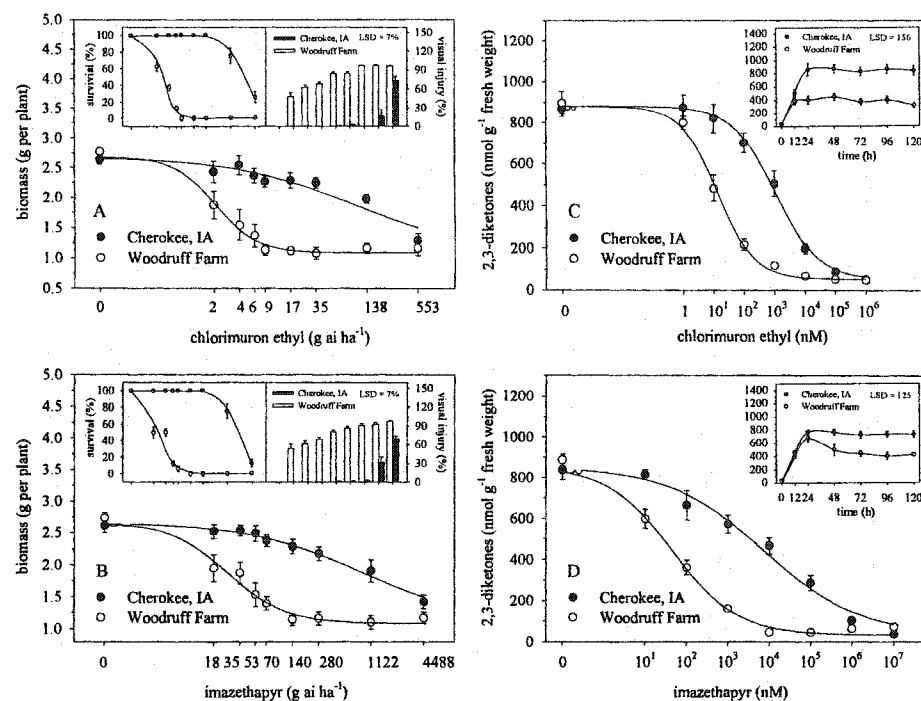
<sup>b</sup> = herbicide dose in g ai ha<sup>-1</sup> that inflicted 50% mortality in the population of the target species.

<sup>c</sup> = herbicide dose in nM that reduced 50% *in vivo* ALS activity in the target species.

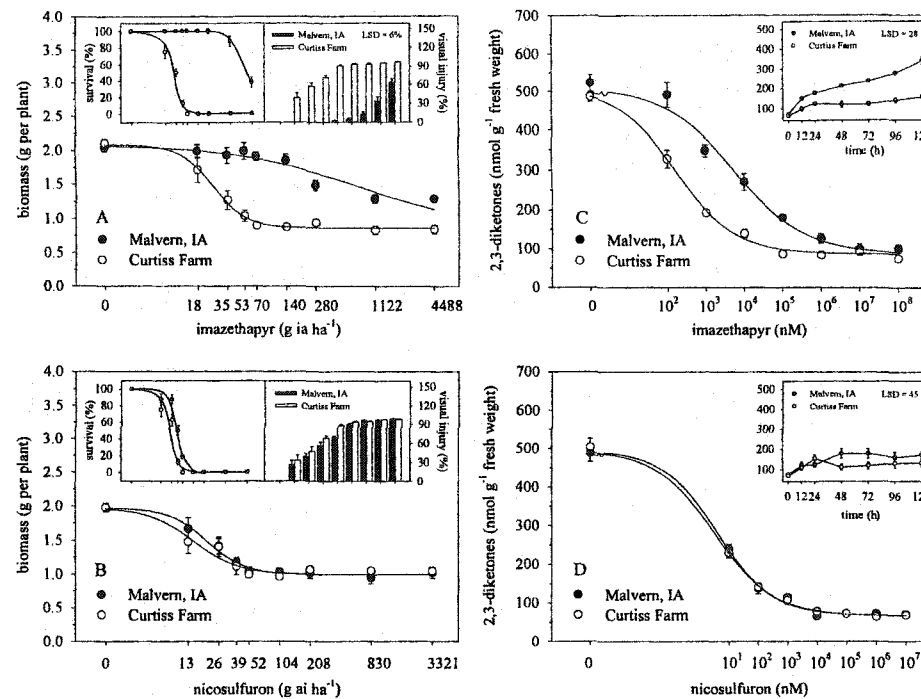
<sup>d</sup> = resistance to susceptibility ratio for the estimated  $GR_{50}$ ,  $LD_{50}$ , and  $I_{50}$  values ( $RS_{GR:LD:I}$ ) calculated by dividing the  $GR_{50}$ ,  $LD_{50}$ , or  $I_{50}$  of the resistant biotype by that of the susceptible biotype.



**Figure 1.** Whole-plant dose (A & B) and *in vivo* ALS (C & D) response of the giant ragweed biotypes (Werner Farm, ●; Zearing, IA, ○) to cloransulam (A & C) and primisulfuron + prosulfuron (B & D). A & B inserts: survival and visual herbicide injury ratings. X-axes in inserts correspond to the herbicide rates in the main graph.  $LSD_{\alpha=0.05}$  values designate statistical differences between two visual injury means at identical herbicide rate. C & D inserts: 2,3-diketone accretion as a function of time; values in the y-axes correspond to the units in the main graph. Cloransulam and primisulfuron + prosulfuron were applied at 10 and 10 + 3.7 nM, respectively.  $LSD_{\alpha=0.05}$  values designate statistical differences between two means at identical sampling time. Symbols or bars in graphs represent the mean of four replications and two experiments; standard errors associated with individual means are depicted in bars.

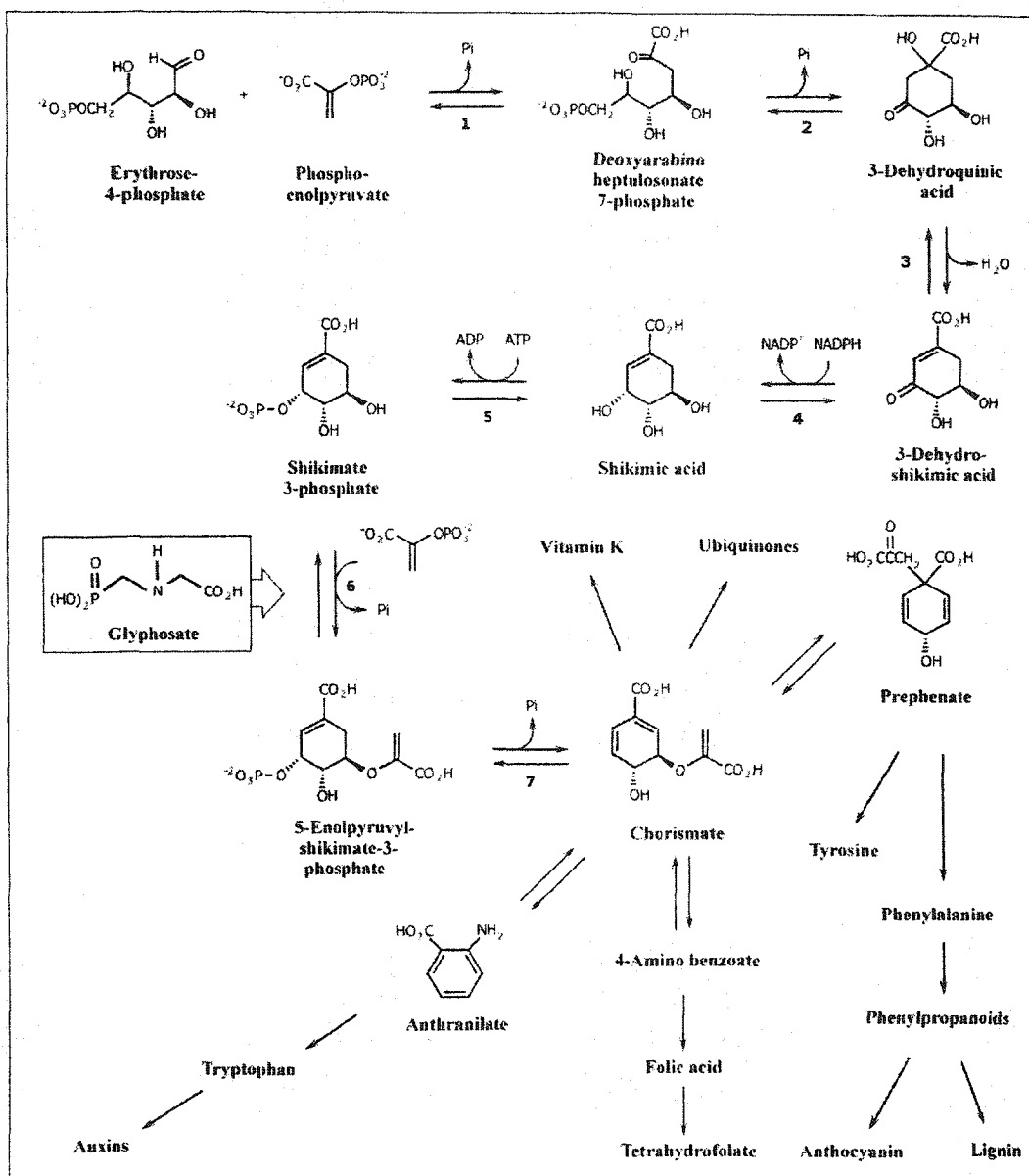


**Figure 2.** Whole-plant dose (A & B) and *in vivo* ALS (C & D) response of the common sunflower biotypes (Cherokee, IA, ●; Woodruff Farm, ○) to chlorimuron (A & C) and imazethapyr (B & D). A & B inserts: survival and visual herbicide injury ratings. X-axes in inserts correspond to the herbicide rates in the main graph.  $LSD_{\alpha=0.05}$  values designate statistical differences between two visual injury means at identical herbicide rate. C & D inserts: 2,3-diketone accretion as a function of time; values in the y-axes correspond to the units in the main graph. Chlorimuron and imazethapyr were applied at 10 and 100 nM, respectively.  $LSD_{\alpha=0.05}$  values designate statistical differences between two means at identical sampling time. Symbols or bars in graphs represent the mean of four replications and two experiments; standard errors associated with individual means are depicted in bars.



**Figure 3.** Whole-plant dose (A & B) and *in vivo* ALS (C & D) response of the shattercane (Malvern, IA, ●; Curtiss Farm, ○) biotypes to imazethapyr (A & C) and nicosulfuron (B & D). A & B inserts: survival and visual herbicide injury ratings. X-axes in inserts correspond to the herbicide rates in the main graph.  $LSD_{\alpha=0.05}$  values designate statistical differences between two visual injury means at identical herbicide rate. C & D inserts: 2,3-diketone accretion as a function of time; values in the y-axes correspond to the units in the main graph. Imazethapyr and nicosulfuron were applied at 1000 and 10 nM, respectively.  $LSD_{\alpha=0.05}$  values designate statistical differences between two means at identical sampling time. Symbols or bars in graphs represent the mean of four replications and two experiments; standard errors associated with individual means are depicted in bars.

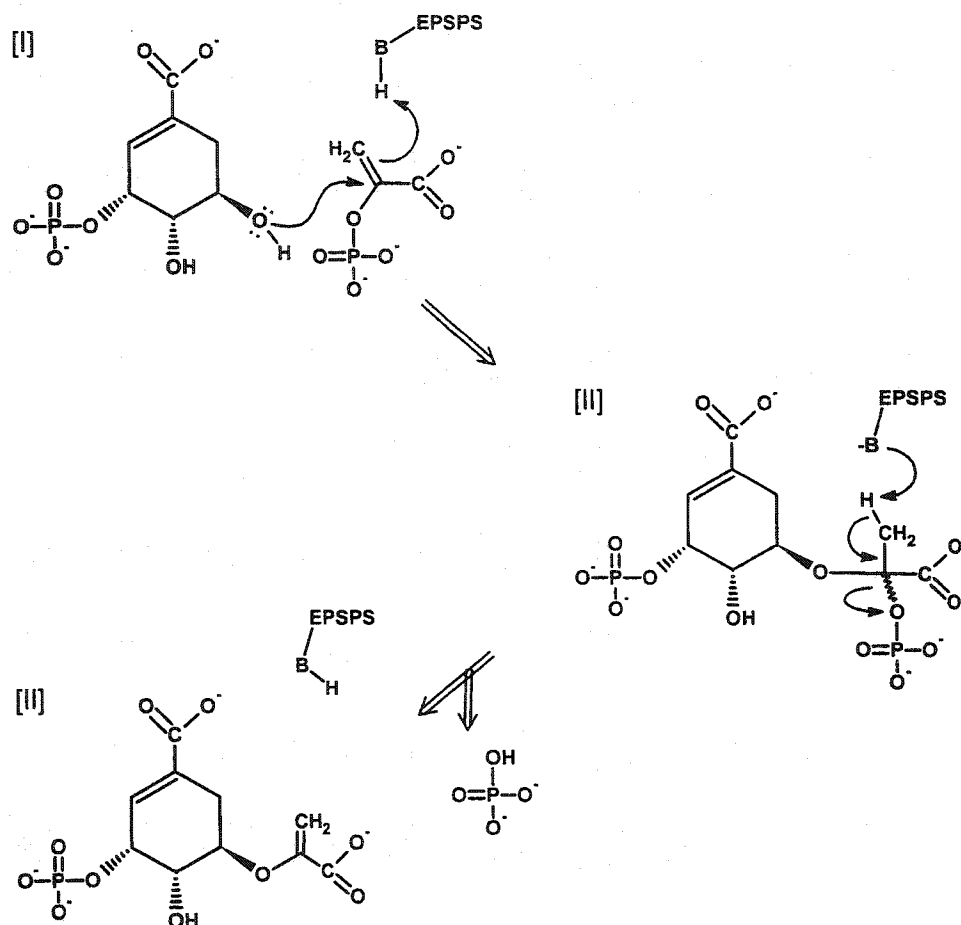
## APPENDIX 2. LINK OF THE SHIKIMIC ACID PATHWAY AND SECONDARY METABOLISM IN PLANTS



**Appendix 2.** The shikimate pathway and byproducts: (1) 2-dehydro-3-deoxyphosphoheptonate aldolase (EC 4.1.2.15), (2) 3-dehydroquinate synthase (EC 4.6.1.3), (3) 3-dehydroquinate dehydratase (EC 4.2.1.10), (4) shikimate 5-dehydrogenase (EC 1.1.1.25), (5) shikimate kinase (EC 2.7.1.71), (6) 3-phosphoshikimate 1-carboxyvinyltransferase (EC 2.5.1.19), and (7) chorismate synthase (EC 4.6.1.4).



**APPENDIX 3. ACCEPTED MECHANISM OF 3-  
PHOSPHOSHIKIMATE 1-CARBOXYVINYLTRANSFERASE (EPSPS,  
EC 2.5.1.19)**



**Appendix 3.** Purported kinetic ESPSP model. [I] nucleophilic attack of the C<sub>5</sub>-OH in 3-phosphoshikimate (3PS) on the C<sub>β</sub> of phosphoenolpyruvate (PEP), [II] formation of a stable tetrahedral intermediate. A base (B<sup>-</sup>) in EPSPS instigates a rearrangements of vinylic protons in PEP, thus the unshared electron pair is donated towards the ester bond in the phosphoryl group of PEP, [III] oxidation of the C<sub>β</sub>-O bond in PEP, regeneration of the base in EPSPS, and release of 5-O- (1-carboxyvinyl)-3-phosphoshikimate (EPSP) plus inorganic phosphate (P<sub>i</sub>).

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